REMARKS

Claims 1, 2, 5 and 11-17 are pending with claims 11-17 being withdrawn. The amendments to the claims are supported in the specification as follows: claim 1: (canceled claims 4 and 9); claim 2: (canceled claim 9); and claim 5: (canceled claim 9). No new matter has been added.

Specification

The disclosure is objected to because it contains an embedded hyperlink and/or other form of browser-executable code. (Office Action p.3)

The hyperlink has been deleted making this objection now moot.

Claim Objections

Claim 1 is objected to because of the following informalities: Claim 1 contains parentheses in lines 4 and 5. (Office Action, Page 4)

The parentheses in claim 1 have been deleted making this rejection now moot.

Regarding claim 3, the phrase "or the like" renders the claim(s) indefinite because the claim(s) include(s) elements not actually disclosed (those encompassed by "or the like"), thereby rendering the scope of the claim(s) unascertainable. (Office Action, Page 4)

Claim 3 has been canceled making this rejection now moot.

Claims 4-8 recites the limitation "the pathological condition" in the 2nd line of each claim. There is insufficient antecedent basis for this limitation in the claim; claim 1 (from which claims 4-8 depend) does not contain reference to "a pathological condition." (Office Action, Page 4)

Claims 4 and 6-8 have been canceled making this rejection moot with respect to these claims. The phrase "the pathologic condition" has been removed from claim 5 and the phrase

"pathologic conditions" has been added to claim 1, thus making this rejection moot with respect to claim 5.

Claims 1-10 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a transgenic mouse model for human arthritis whose a genome comprises a foreign DNA consisting of MHC class II transactivator gene, under the control of a type II collagen promoter wherein said mouse, does not reasonably provide enablement for the broad genus of transgenic animals having genetic mutations as described in claim 1. (Office Action, Page 5)

Claims 1, 2 and 5 have been amended to recite a transgenic mouse or rat and the remaining claims have been canceled. In view of the teachings in the instant specification and the state of the art, a person skilled in the art can make the invention set forth in the amended claims without undue experimentation.

The present invention is based on the finding that by carrying out gene modification so that the CIITA gene is expressed by the effect of a type II collagen promoter, a model animal that well represents pathologic conditions of human rheumatoid arthritis can be produced is obtained (see [0005]). The function of this gene is well recognized (see [0008]). A person skilled in the art would appreciate that not completeness of the sequence but the function of the gene is essential to the invention. The function as a master switch for controlling an expression of this gene is well recognized in the art (see enclosed Annu. Rev. Immunol, 2001, 19:331-73 and Eur. J. Immunology, 34, 1513-1525, 2004). The function is encoded by the active region which is also well recognized in the art (see Cell, 109, S21-S33, 2002 and International Immunology, 13, 951-958, 2001).

Both of type II collagen promoter and MHC class II transactivator gene are highly conserved beyond differences of species. Thus, it is logical to understand that these two play the same roles in a mouse and a rat and the technical feature of the present invention which employs the combination of these two elements brings the same result, namely the production of an excellent animal model for human arthritis. This is supported by the fact that a number of past studies have proved that in most cases the same genetic modifications bring the same results in a mouse and a rat. Thus, predictability of success in a rat is very high.

As a result, an art skilled person can make the invention set forth in the amended claims without undue experimentation. It is respectfully requested that the rejection be reconsidered and withdrawn.

Claims 1-2 are rejected under 35 USC 103(a) as being unpatentable over Fabre et al. (WO98/15626) in view of Osaki et al. (Biochemical Journal. 11 September 2002; 1-34). (Office Action p.16)

As will be shown below, the combination of the elements are not disclosed nor suggested by the combination of Fabre in view of Osaki. Further the combination of the prior art would not at all suggest the unexpected results of the claimed invention.

In general, predictability in this art is low. Unless any findings supported by actual experiments, even the skilful artisan cannot foresee the result of any combination. The present inventors employ the combination of type II collagen promoter and MHC class II transactivator gene to express this gene in cartilage. Whether or not the genetic modification using the combination would bring success in producing a desired animal model was unpredictable. Actually, forced expression of MHC class II transactivator gene alone did not show any phenotype similar to human rheumatoid arthritis spontaneously. Surprisingly, the transgenic mouse showed pathologic conditions of human rheumatoid arthritis by the induction using a very small amount of type II collagen such as 0.01 mg to 0.05 mg. Namely, it was revealed that the transgenic mouse acquired a high susceptibility to a foreign antigen. In an H-2^q haplotype mouse that has been conventionally used as a model animal of human rheumatoid arthritis, *such a small dose amount of the type II collagen cannot induce the pathologic conditions of human rheumatoid arthritis satisfactorily.* The present invention would never be achieved without this *unexpected result.*

The transgenic mouse or rat of the invention shows excellent features in comparison with conventional animal models as described in the specification. Relevant portions are quoted (emphasis added):

> In other words, the TG animal of the present invention has ***higher sensitivity showing the pathologic conditions*** of human rheumatoid arthritis than the H-2^q

haplotype mouse that has been conventionally used as a model animal of human rheumatoid arthritis. [0097]

As a result, in general CIA, in several days after the secondary immunization is carried out, strong redness and swelling are observed. While in the transgenic mouse, redness and swelling in the extremities have been observed for several weeks or more (FIG. 2). [0157]

When about two months had passed, the swelling in the extremities progressed further. Although some individuals showed slight remission, basically bone destruction progresses, so that disorders such as bone deformity were observed finally (FIG. 6). [0158]

In a conventional CIA method, at the fifth week following the immunization, the change of the bone density was observed, and large change was not shown thereafter. On the other hand, in the ***transgenic mouse, not only the progress of inflammation but also the change in the bone density progresses gradually.*** [0159]

For example, primary lesion of the respiratory organ, vasculitis, reduction of red blood cell count, which are caused by rheumatism, are confirmed. [0160]

The combination of references do not at all suggest the unexpected result of *such a small dose amount of the type II collagen.* Thus it the combination of references fails to create a *prima facie* conclusion of obviousness teaching the invention as now claimed.

If this showing is not enough, PNAS, September 26, 2006, vol.103, no.39, 14465-14470 is attached. FIG. 1A and the description on p.14466, *left column lines 4-9 support the unexpected nature of the result explained above.*

Based upon this showing, it is respectfully requested that the rejection be reconsidered and withdrawn.

Claims 1-10 are rejected under 35 USC 103(a) as being unpatentable over Harton et al. (Molecular and Cellular Biology, Sept. 2000; 20(17):6185-6194) in view of Lindqvist et al. (Trends in Genetics. 2002; S7-S13) and further in view of Otten et al. (Journal of Immunology. 2003; 170: 1150-1157). (Office Action p.19)

It is admitted in the rejection that Harton, Lindqvist and Otten do not teach a transgenic non-human mammal comprising CIITA operably linked to collage II promoter. For the reasons above with respect to remaining claims 1, 2 and 5, the combination of references fails to suggest the *unexpected results achieved by the invention as now claimed.* Therefore, there is no obvious predictability, as asserted by the rejection, created by the combination of the three references with respect to the *claimed invention.* Thus it is respectfully requested that the rejection be reconsidered and withdrawn.

In view of the above amendment, applicant believes the pending application is in condition for allowance. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105.

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Customer No. 21874

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THE BARE LYMPHOCYTE SYNDROME AND THE REGULATION OF MHC EXPRESSION

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Key Words MHC class II deficiency, primary immunodeficiency disease, transcription regulation, CIITA, RFX

■ **Abstract** The bare lymphocyte syndrome (BLS) is a hereditary immunodeficiency resulting from the absence of major histocompatibility complex class II (MHCII) expression. Considering the central role of MHCII molecules in the development and activation of $CD4^+$ T cells, it is not surprising that the immune system of the patients is severely impaired. BLS is the prototype of a "disease of gene regulation." The affected genes encode RFXANK, RFX5, RFXAP, and CIITA, four regulatory factors that are highly specific and essential for MHCII genes. The first three are subunits of RFX, a trimeric complex that binds to all MHCII promoters. CIITA is a non-DNA-binding coactivator that functions as the master control factor for MHCII expression. The study of RFX and CIITA has made major contributions to our comprehension of the molecular mechanisms controlling MHCII genes and has made this system into a textbook model for the regulation of gene expression.

PREAMBLE: BLS as a Unique Model System

Major histocompatibility complex class II (MHCII) deficiency (official WHO nomenclature) is frequently also referred to as the bare lymphocyte syndrome (BLS). It is a rare form of inherited immunodeficiency having an autosomal recessive mode of inheritance. The disease is characterized by the lack of expression of MHCII molecules, which leads to severe immunodeficiency, recurrent infections, and frequently to death in early childhood (1–5). BLS exhibits several remarkable and unique features that have fascinated geneticists, immunologists, and specialists in the regulation of gene expression.

From the point of view of medical genetics, BLS is of special interest for at least three reasons. First, the genes implicated in the phenotypic manifestation of the disease, namely the family of MHCII genes on chromosome 6, are in fact intact. The genes that are mutated in BLS are distinct from the MHCII genes themselves and are located on different chromosomes (Figure 1). Mutations in

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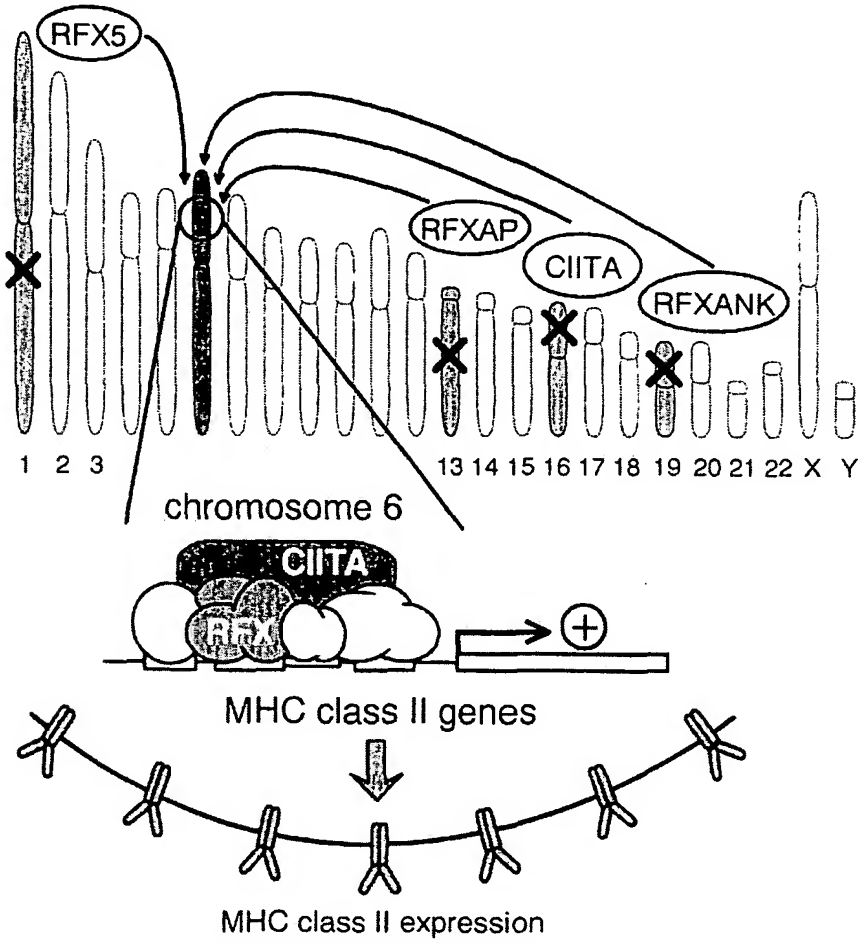


Figure 1 The bare lymphocyte syndrome (BLS) is a disease of MHCII gene regulation. BLS is characterized by a dissociation between the genes that are mutated in the disease (*top: RFX5, RFXAP, CIITA* and *RFXANK* on chromosomes 1, 13, 16 and 19, respectively) and the genes whose lack of expression accounts for the observed phenotype (*bottom*, MHCII genes on chromosome 6).

any one of these non-MHC genes are responsible for the lack of MHCII expression. This dissociation between the genes that are defective in the disease and those that are implicated in the observed phenotype is highly unusual (Figure 1). Second, although BLS is a monogenic disease in which a single defective gene is responsible for the entire clinical picture, and although the disease is clinically homogeneous, it can result from mutations in any one of four distinct genes (Figure 1). Curiously, a genetic defect in each of these four genes leads to the same clinical syndrome. This genetic heterogeneity associated with clinical homogeneity is also unusual. Thirdly, BLS represents the prototype of a "disease of gene regulation." The affected genes encode four *trans*-acting regulatory factors that are essential and highly specific for the control of MHCII gene expression (Figure 1).

It is evident that the last point explains the aforementioned distinction between the genetic defects and their phenotypic manifestations.

From the point of view of the control of the immune response, as well as of the regulation of gene expression in general, elucidation of BLS at the molecular level has led to numerous seminal findings. First, it is fair to say that the discovery of the four regulatory genes implicated in BLS has represented a major contribution to what we know today about the molecular basis of the regulation of MHCII gene expression. Given the tight and complex nature of this regulation, and its central role in the control of the immune response, the contribution of BLS to immunology has been remarkable. Second, identification of the four regulatory genes affected in BLS has provided us with a unique example of four transcription factors that are both essential and specific for the control of MHCII expression. This also represents a rather unusual situation because most transcription factors exhibit functional redundancy with other factors and/or exert pleiotropic effects via their control over numerous unrelated target genes. Finally, dissection of the mechanisms by which the four regulatory factors affected in BLS exert their control over MHCII genes has provided us with a texbook model for the regulation of gene expression. In particular, it has emphasized the essential role of protein-protein interactions in transcriptional control and the importance of cooperativity as a means for generating specificity.

The first part of this review covers BLS as a disease, discusses its molecular basis, and summarizes the discovery, between 1993 and 1998, of the four affected regulatory genes and the transacting factors that they encode. The second part deals with the contribution that the discovery of these four transactivators has made to our current understanding of the mechanisms regulating the expression of MHCII genes.

INTRODUCTION: Function and Regulation of MHCII Expression

MHCII molecules are heterodimeric (α/β) transmembrane glycoproteins. In humans there are three MHCII isotypes—HLA-DR, HLA-DQ, and HLA-DP—each of which is composed of a distinct pair of α and β chains. The genes encoding the α and β chains of HLA-DR, HLA-DQ, and HLA-DP are clustered in the D region of the MHC on the short arm of chromosome 6 (6). MHCII molecules are specialized for the presentation of peptides to the antigen receptor (TCR) of $CD4^+$ T helper cells. Engagement of MHCII–peptide complexes by the TCR of $CD4^+$ T cells is a key event in the development, activation, and regulation of the adaptive immune system. First, the recognition of MHCII–peptide complexes on epithelial cells and bone marrow–derived cells in the thymus is central to the positive and negative selection processes that sculpt the TCR repertoire of the $CD4^+$ T cell population (7). Second, the priming, propagation, and regulation of antigen-specific immune responses by $CD4^+$ T cells requires the interaction

of their TCR with MHCII–peptide complexes displayed on antigen presenting cells (APC) (8). These APC are specialized for the MHCII-mediated presentation of peptides derived from the internalization and processing of exogenous protein antigens. Finally, the life span of $CD4^+$ T cells in the periphery is influenced by interactions with cells expressing MHCII molecules (7). Given these key functions of MHCII molecules, it is not surprising that defects in their expression have severe immunopathological consequences. The inability to express MHCII molecules leads to a severely crippled immune system that is incapable of responding adequately to foreign antigens (1–5). In contrast, aberrant or inappropriate MHCII expression has been incriminated in certain $CD4^+$ T cell–mediated autoimmune diseases (9). These two points emphasize the importance of correctly regulated MHCII expression for the control of the immune response in health and disease. For nearly two decades, a detailed elucidation of the molecular mechanisms that regulate MHCII expression has therefore represented a major challenge in molecular immunology.

Constitutive MHCII expression is generally restricted to a small number of cells of the immune system (4, 10–13). These include primarily bone marrow–derived APCs, namely dendritic cells (DCs), B cells, and cells of the monocyte/macrophage lineage. MHCII expression is also characteristic of epithelial cells in the thymus. Finally, in humans, activated T cells express MHCII. Constitutive expression in all three APC lineages is regulated as a function of developmental stage. Maturation of DCs is accompanied by an increase in cell surface MHCII expression. Activation of macrophages by stimuli such as interferon gamma (IFNγ) also leads to enhanced MHCII expression. In contrast, MHCII expression is extinguished upon differentiation of B cells into plasma cells.

The majority of non–bone marrow–derived cell types generally do not express MHCII molecules. However, MHCII expression can be induced in these cells by a variety of stimuli, of which IFNγ is most potent and well known. IFNγ–induced MHCII expression can be further modulated by a diverse array of other influences. For example, TGFβ, IFNβ, TNFα, IL-1, infection by a variety of pathogens, and certain drugs can attenuate or block the induction of MHCII expression by IFNγ.

Both constitutive and IFNγ–induced MHCII expression are controlled primarily at the level of transcription by a highly conserved regulatory region situated within the first 150 base pairs upstream of the transcription initiation site (4, 10–13). This promoter proximal regulatory region consists of four *cis*-acting elements referred to as the S (also called W or Z), X, X2, and Y boxes. These four elements are highly conserved in their sequence, orientation, order, and spacing relative to each other, and they function together as a single composite MHCII regulatory module (4, 11–13). The same architecture is evident in the promoters of all MHCII genes from every species that has been examined. A similar arrangement has also been conserved in the promoter regions of the MHCII-related *Ii*, *HLA-DM*, and *HLA-DO* genes (14–19), which code for proteins implicated in the intracellular traffic and peptide loading of MHCII molecules (20–22). Finally, it has recently also been appreciated that a region resembling the MHCII regulatory module

contributes, albeit to a lesser extent, to expression of the MHC class I (MHCI) and $\beta 2$ microglobulin (*B2M*) genes (23–27).

With the aim of dissecting the molecular mechanisms regulating transcription of MHCII genes, a considerable amount of effort has been devoted to the identification of transcription factors that bind to the MHCII regulatory module. Over 20 different nuclear factors capable of binding in vitro to the S, X, X2, and Y boxes were identified and/or cloned (4, 10–13). This complexity led to considerable confusion and controversy regarding the functional relevance and importance of all these MHCII promoter-binding factors. Distinguishing the factors that control transcription of MHCII genes from those that are functionally irrelevant represented a formidable task. This problem was solved to a large extent by a genetic approach. This genetic approach relied on the elucidation of the molecular defects underlying the absence of MHCII expression in the bare lymphocyte syndrome (BLS). Isolation of the genes that are defective in BLS has led to the unequivocal identification of CIITA and the multiprotein RFX complex, two key regulatory factors that activate transcription of the genes encoding the MHCII, HLA-DM, HLA-DO, Ii, and MHCI molecules (28–34).

PART 1: MHCII Deficiency or the Bare Lymphocyte Syndrome

Clinical Manifestations and Pathology

Patients suffering from a primary immunodeficiency syndrome characterized by a defect in MHC expression were first reported in the late 1970s and early 1980s (35–40). Curiously, the term BLS (41) was initially used to describe a reduced level of MHCI molecules in patients in which MHCII expression was not examined. Since then, a constant and profound defect in MHCII expression has been recognized as the major cause of the syndrome, and the disease was thus formally named MHCII deficiency by the World Health Organization (42). However, the term BLS is still widely used as a synonym for MHCII deficiency. The disease has been assigned the MIM (Mendelian Inheritance in Man) number 209920.

BLS is a rare autosomal recessive disease. Fewer than 80 patients coming from about 60 unrelated families have been formally reported worldwide. As expected for a rare inherited disease, there is a high incidence of consanguinity in the affected families (43). A majority of the affected families are from North Africa, but Turkey and Spain are also well represented (1, 3, 43).

The lack of expression of MHCII antigens results in a severe defect in both cellular and humoral immunity, and the patients thus exhibit an extreme vulnerability to infections (1–3, 5). This includes viral, bacterial, and fungal as well as protozoal infections. Clinical manifestations typical of BLS include mainly recurrent infections of the gastrointestinal tract, pneumonitis, and bronchitis. Severe septicemia is also common. Multiple infectious agents are responsible for the infections.

Pseudomonas and Salmonella, as well as cytomegalovirus, are found frequently. Major clinical findings resulting from bacterial infections of the gastrointestinal tract are protracted diarrhea, malabsorption, and failure to thrive. Infections start within the first year of life, and there is a dramatic progression of various types of infectious complications, generally leading to death before the age of 10. All of the clinical manifestations of the disease are related to infections and presumably result from the lack of MHCII expression. From a clinical point of view, no distinction has been made between BLS patients belonging to the four genetic complementation groups (see below). The most common clinical manifestations of BLS are summarized in Table 1. A more detailed presentation of the clinical picture of BLS can be found elsewhere (1–3, 5).

Laboratory Findings and Immunological Features

The most striking and constant immunological feature of BLS is the absence of cellular and humoral immune responses to foreign antigens (1–3, 5). Interestingly, all of the immunological features and anomalies recognized in BLS patients can be accounted for by the lack of MHCII expression and by its consequences in terms of antigen presentation. Surprisingly, the immunological anomalies exhibited by BLS patients, including their laboratory parameters, are notoriously variable from patient to patient. However, as observed for the clinical manifestations, no correlation has been recognized with the four distinct genetic groups of patients defined by the individual regulatory genes that are affected (see below).

Detailed accounts of the immunological features and laboratory findings typical of BLS have been published previously (1–3, 5). Patients are unable to mount T cell–mediated immune responses in vivo, and their T cells are not activated in vitro by antigens to which the patients have been exposed. Humoral immune responses are also severely affected: Hypogammaglobulinemia is characteristic, and antibody responses to immunizations and infections are reduced or absent (Table 1). The total numbers of circulating T and B lymphocytes is normal, but the number of $CD4^+$ T lymphocytes is reduced (Table 1). The severity of this reduction varies from patient to patient. The remaining $CD4^+$ T lymphocytes do not exhibit major abnormalities in their TCR repertoire, and they seem to be functionally normal as judged by alloreactivity and responses to mitogens. Their physiological responses to MHCII mediated antigen presentation have however not been studied thoroughly. The presence of a significant number of residual $CD4^+$ T lymphocytes in patients that fail to express MHCII molecules remains an interesting paradox. Residual expression of MHCII molecules in the thymus, although not clearly documented, or alternative pathways for positive selection of $CD4^+$ T cells, have been mentioned as possible explanations (see below).

Abnormal Expression of MHCII Genes

Since the initial descriptions of the disease, understanding the cause of BLS has represented a challenge for immunologists interested in the crucial role of MHCII and of its remarkably tight regulation in the control of the immune response.

TABLE 1 Clinical manifestations and immunological features of BLS

Clinical manifestations	Fraction of patients[1]
Repeated severe infections	47/47
Protracted diarrhea	45/47
Lower respiratory tract infections	40/47
Failure to thrive	34/47
Severe viral infections	27/47
Upper respiratory infections	24/47
Mucocutaneous candidiasis	16/47
Progressive liver disease	8/47
Cryptosporidiosis	8/47
Autoimmune cytopenia	4/47
Sclerosing cholangitis	5/47
Immunological findings	**Fraction of patients[1]**
Complete absence of MHCII expression:	
B cells	36/37
monocytes	31/37
PHA activated T cells	32/37
Residual MHCII expression:	
monocytes	6/37
PHA activated T cells	5/37
Reduction of MHCI expression:	
mononuclear cells	23/30
CD4+ lymphopenia	28/31
Serum immunoglobulins:	
decreased IgG	18/22
decreased IgM	23/32
decreased IgA	25/32
Decreased or absent antibody response to:	
immunizations	15/16
microbial antigens	26/26

[1]data adapted from Reith et al., 1999 (2).

Numerous research groups therefore turned to the study of the molecular basis of BLS as a way to learn more about the regulation of MHCII expression. Here we describe the progress that was made toward this goal in a chronological order, as a succession of logical experimental milestones. We are very grateful for the long-term collaborations between our laboratory and those who provided the BLS cell lines that made our studies possible, particularly the laboratories of C Griscelli (Paris) and M Hadam (Hanover).

As described in the Introduction, two distinct modes of MHCII expression are normally observed in healthy individuals: constitutive expression in a restricted number of highly specialized cells, and inducible expression in response to specific stimuli, particularly IFNγ. In BLS, both constitutive and inducible expression of MHCII antigens abolished (1–3, 5). The lack of cell surface MHCII molecules is evident on all professional antigen-presenting cells, including B cells, macrophages such as Kupfer cells in the liver, and dendritic cells such as Langerhans cells in the skin. Activated T cells remain MHCII negative. Moreover, cells that are not of bone marrow origin, such as thymic epithelial cells and endothelial cells, also lack MHCII molecules. Finally, cells from the patients do not express MHCII molecules following stimulation with IFNγ.

This general lack of cell surface MHCII expression was confirmed at the level of intracellular MHCII proteins (44). The next step was to show that no MHCII mRNA could be detected in any of the cell types and conditions studied (3, 44–46). It was further shown that the deficiency in expression concerned the α- and β-chain genes coding for the HLA-DR, HLA-DQ, and HLA-DP molecules, implying a general block in the expression of all MHCII genes (3, 44–46). More recently, a partial block in the expression of the MHCII-related genes encoding HLA-DM, HLA-DO, and the Ii chain was also described (15, 47, 48).

Occasional weak residual expression of MHCII antigens and mRNA has been reported in certain BLS patients (Table 1). This residual expression has been described both for constitutive expression in certain cell types and for inducible expression in response to IFNγ (1–3, 5). We have not observed such a leaky phenotype in most of the EBV-transformed BLS B cell lines that we have studied. In addition to the complete extinction of MHCII expression, a relatively minor reduction in the level of MHCI molecules has been observed in BLS (Table 1) (1–3, 5). Again, this reduction in MHCI is not evident, at least at the level of cell surface expression, in most of the typical BLS B cell lines that we have studied.

BLS Is a Disease of Gene Regulation Involving Defects in "Transacting" Factors

The demonstration that BLS is a disease of gene regulation represented a key step in the elucidation of BLS. The fact that all MHCII genes were found to be silent first suggested that there was a general defect acting in *trans* on the entire gene family. To test this hypothesis we studied the affected families for transmission of both the disease phenotype and the MHC locus on chromosome 6 (3, 45). The two clearly did not cosegregate, indicating that the genetic defect(s) responsible for BLS reside outside of the MHC. It was the merit of the late Claude de Préval to publish these findings in a 1985 *Nature* paper entitled "A trans-acting class II regulatory gene, unlinked to the MHC, controls the regulation of HLA class II expression" (45). This statement was formally confirmed 8 years later when we identified and cloned the first *trans*-acting gene affected in BLS (29 see below). Another

important confirmation of the regulatory nature of the BLS defect came from cell fusion experiments demonstrating that MHCII expression can be reactivated upon fusion of MHCII negative cells affected in two different genes (see below) (43, 49–51). Finally, it was subsequently shown that MHCII promoters remain silent in functional studies performed in BLS cells (52, 53) and that nuclear extracts from BLS cells cannot support in vitro transcription from MHCII promoters (54). The realization that BLS is due to defects in regulatory factors specific for MHCII genes suggested that elucidation of the genetic basis of BLS was likely to lead, beyond the scope of the disease itself, to the discovery of genes and factors directly involved in the important but elusive molecular mechanisms controlling MHCII expression.

Genetic Heterogeneity in BLS

The search for the genetic defects underlying BLS became even more exciting and challenging when it was found that the disease is genetically heterogeneous and that patients could be classified into four distinct complementation groups (Table 2, groups A to D). The conclusion that the disease is genetically heterogeneous was drawn from somatic cell fusion experiments performed with MHCII-negative cell lines derived from BLS patients (43, 49–51, 55). In certain combinations MHCII expression was clearly restored, indicating that the two cell lines belong to distinct genetic complementation groups. These studies revealed the existence of four

TABLE 2 Genetic, biochemical and molecular heterogeneity in BLS

BLS complementation group	**A**	**B**	**C**	**D**
Prototypical patients	BLS-2, BCH	BLS-1, Ra	SJO, Ro	DA, ABI
Number of unrelated families	5	26	5	6
Prototypical in vitro mutant	RJ2.2.5	None	G1B	6.1.6
Number of in vitro mutants	3	None	1	1
MHCII expression	Absent	Absent	Absent	Absent
MHCII promoter activity	Absent	Absent	Absent	Absent
Binding of RFX	Yes	No	No	No
Promoter occupancy in vivo	Occupied	Bare	Bare	Bare
Affected gene	*MHC2TA*	*RFXANK*	*RFX5*	*RFXAP*
MIM number	600005	603200	601863	601861
mRNA sequence entry	X74301	AF094760	X85786	Y12812
Chromosomal localization	16p13	19p12	1q21.1–21.3	13q14
Protein	CIITA	RFXANK	RFX5	RFXAP
Length (amino acids)	1′130	269	616	272
Apparent molecular weight	130 kD	33 kD	75 kD	36 kD
No. distinct mutations in BLS	5	7	5	3

complementation groups (43, 49–51,55). It is now known that the majority of patients (26 out of 42 families) belong to group B, while groups A, C, and D correspond respectively to 5, 5, and 6 unrelated families (Table 2) (56). In addition to cells from BLS patients, several in vitro–generated mutant MHCII-negative cell lines have also been allocated to the complementation groups. These include RJ2.2.5 (group A), G1B (group C), and 6.1.6 (group D) (Table 2) (49–51, 57–59). The identification of four genetic complementation groups strongly suggested the existence of genetic loci corresponding to four key MHCII regulatory genes. This greatly strengthened the interest in the genetic and molecular basis of BLS because it implied that elucidation of this rare disease should lead to the discovery of four distinct regulatory genes that are all essential and specific for the control of MHCII expression.

As mentioned earlier, none of the clinical, pathological, or immunological features observed in BLS patients is typical of or unique to one of the four genetic groups. The syndrome is therefore genetically heterogeneous but clinically and phenotypically homogeneous. There is, however, an interesting correlation between individual BLS groups and the ethnic origin of the patients: Patients from group B are predominantly of North African origin while those from group A are mainly Hispanic (43).

Atypical Form of Deficiency in MHCII Expression

A family affected by an atypical and much less severe form of deficiency in MHCII expression has been described (60–62). Defective expression in the two patients from this family does not concern all MHCII genes equally. In B cell lines derived from these two patients, the *HLA-DRB*, *HLA-DQB*, and *HLA-DPA* genes are silent while the *HLA-DRA*, *HLA-DQA*, and *HLA-DPB* genes are expressed (62). Interestingly, the silent genes are present in one transcriptional orientation within the MHCII locus, whereas the active ones are found in the opposite orientation (62). This intriguing discordant pattern of expression remains unexplained. Impairment of the immune response is considerably less severe in the two atypical patients than in classical BLS (60, 61). This has been suggested to be due to the fact that the deficiency in MHCII expression does not affect all cell types to the same extent. Indeed, investigations of MHCII-dependent immune functions in these patients indicate the presence of competent MHCII positive APCs (60, 61). As expected from their unusual phenotype, the atypical patients represent a distinct medical and genetic entity compared to the classical BLS groups A to D (62). The molecular defect underlying this atypical form of immunodeficiency has not yet been elucidated.

DNA-Binding of RFX Distinguishes Between Two Types of Molecular Defect in BLS

RFX (Regulatory Factor X) is a DNA-binding protein complex first identified in MHCII positive B cells on the basis of its ability to bind in vitro to the X box of MHCII promoters (30). Binding activity of the RFX complex was studied

extensively with nuclear extracts prepared from various wild-type and BLS cell lines (30, 54, 63). It was recognized rather early that two types of BLS defects could be defined on the basis of the presence of the RFX complex (Table 2). Binding of RFX was found to be normal in wild-type cells as well as in cells from BLS patients in group A. On the other hand, cells from patients in groups B, C, and D were specifically devoid of RFX binding activity. This observation indicated that the genetic defects in groups B–D affect the integrity or binding activity of the RFX complex, while the defect in group A is independent of RFX.

Interestingly, there is a tight correlation between the status of RFX binding activity in vitro and the state of occupancy of MHCII promoters in vivo (Table 2). This was analyzed either by mapping of DNAseI hypersensitive sites (64) or by in vivo footprint experiments (65, 66). BLS cells lacking a functional RFX, complex (groups B, C and D) exhibit a "bare" promoter phenotype in which the X, X2, and Y sequences of MHCII promoters are not bound by their cognate DNA-binding proteins (65, 66). In contrast, wild-type cells as well as cells from BLS patients in group A exhibit both normal RFX binding activity in vitro and normal MHCII promoter occupancy in vivo (65, 66). The fact that not only the X box, but also the X2 and Y boxes are unoccupied in vivo in the absence of RFX, points to a crucial role of RFX in facilitating overall promoter occupation. It is now clear that this is explained by the fact that RFX entertains strong cooperative binding interactions with other MHCII promoter binding factors (67–71), including the Y box binding factor NF-Y (72) and the X2 box binding factor X2BP (67). The importance of these cooperative protein-protein interactions in the specificity of gene activation is discussed further below.

CIITA: The Regulatory Gene and Factor Affected BLS Group A

Paradoxically, although the deficiency in RFX offered an experimental approach toward the identification and cloning of the genes affected in BLS groups B–D, the first BLS gene and factor to be discovered was the one affected in group A (29). The strategy employed relied on the genetic complementation of RJ2.2.5, one of the in vitro generated cell lines classified in group A. Following transfection of RJ2.2.5 with a B cell cDNA library prepared in an episomal expression vector, plasmids capable of correcting the genetic defect were rescued from cells in which MHCII expression was restored. This approach led to the isolation of cDNA clones encoding the MHC class II transactivator CIITA (Figure 2) (29). The human gene (*MHC2TA*) encoding CIITA is localized on chromosome 16 (16p13) (Figure 1). The mouse gene (*Mhc2ta*) is situated in a syntenic region of mouse chromosome 16 (V Steimle, B Mach, unpublished data). This localization is consistent with previous experiments demonstrating that regulatory genes present on the human and mouse chromosomes 16 (the *AIR-1* locus) are required for expression of MHCII genes (73, 74). The entire intron-exon structure of the mouse *Mhc2ta* gene has been determined. It consists of 19 exons spanning 42 kb of genomic DNA

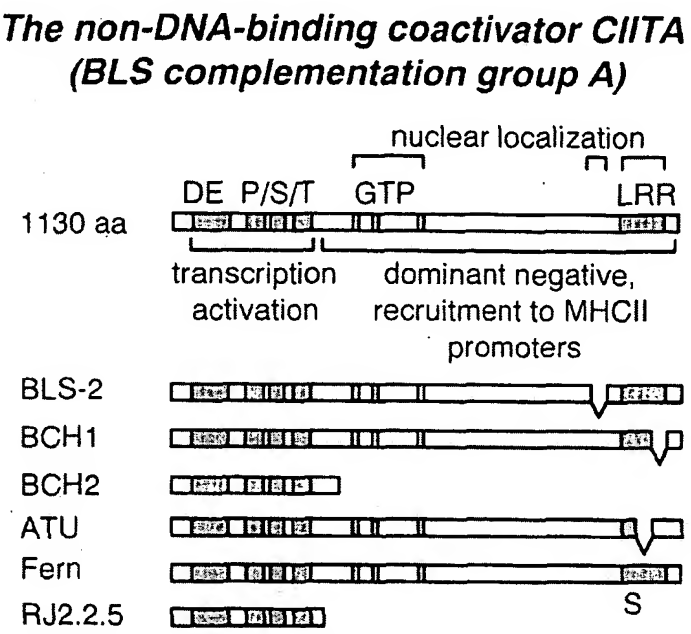


Figure 2 Structure, function, and mutation of CIITA, the regulatory factor that is affected in BLS complementation group A. A schematic map of the CIITA protein is shown at the top. The three sequences constituting a GTP binding domain, the leucine-rich repeat domain (LRR), the acidic region (DE), and 3 segments rich in proline, serine, and threonine (P/S/T) are represented by grey boxes. Regions that function in activation of transcription, in recruitment to MHCII promoters, in nuclear localization, and as a dominant negative mutant are indicated. Mutations affecting the *MHC2TA* gene have been determined in four BLS patients (BLS-2, BCH, ATU, and Fern) and in the in vitro generated cell line RJ2.2.5. The effects of the mutations at the level of the CIITA protein are represented schematically.

(W Reith, B Mach, unpublished data). Defects in the *MHC2TA* gene account for patients in complementation group A (Figure 2). Expression vectors encoding CIITA can reactivate expression of all three MHCII isotypes when transfected into cell lines from complementation group A (29). Wild-type levels of *Ii*, *HLA-DMA*, and *HLA-DMB* expression are also restored (47, 75). Five different mutations of the *MHC2TA* gene have been characterized in BLS patients from group A (29, 56, 76–78). Both alleles of the *MHC2TA* gene are also mutated in RJ2.2.5 (29, 79).

The Genes Encoding RFX, the DNA-Binding Complex Affected in BLS Groups B to D

Purification of RFX demonstrated that it consists of three subunits having apparent molecular weights of 75, 36, and 33 kDa (33, 54, 80). This suggested that defects in the genes encoding these three subunits were likely to account for the absence of RFX binding activity in cells from BLS groups B, C, and D. Isolation of the genes encoding the three subunits of RFX confirmed this interpretation (31–34).

We called these genes *RFXANK*, *RFX5*, and *RFXAP* and demonstrated that they are indeed mutated in BLS complementation groups B, C, and D, respectively (Table 2, Figure 3) (31–33).

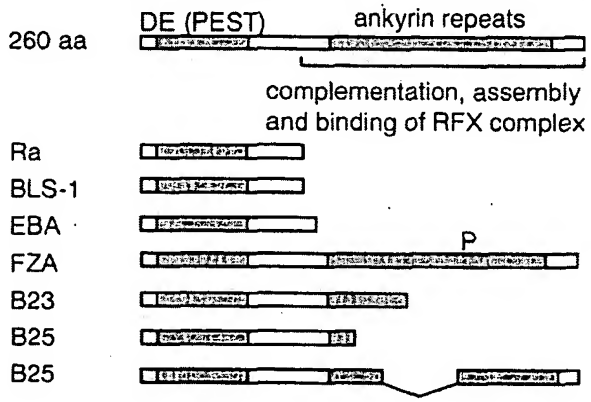
RFX5: The Regulatory Gene and Factor Affected in BLS Group C *RFX5*, the gene encoding the largest (75 kDa) subunit of the RFX complex, was isolated by the same genetic complementation approach that led to the cloning of CIITA (32). Briefly, *RFX5* cDNA clones were isolated by virtue of their ability to restore MHCII expression upon transfection into SJO, a cell line derived from a BLS patient in complementation group C (32). RFX5 derives its name from the fact that it is the fifth isolated member of a family of DNA-binding proteins capable of recognizing the MHCII X box (81). All members of this RFX family share a common motif called the RFX DNA-binding domain (Figure 3) (81, 82). The human *RFX5* gene is situated in a subcentromeric region of the long arm of chromosome 1 (1q21.1–21.3) (83). The corresponding mouse gene maps to a syntenic region of chromosome 3 (W Reith, unpublished data). The entire intron-exon structure of the mouse *Rfx5* gene has been determined and shown to consist of 10 exons (W Reith, unpublished data). Defects in *RFX5* account for the existence of complementation group C (Figure 3). Mutations in *RFX5* have been characterized in five patients from group C (32, 56, 83–85). A missense mutation has also been identified in G1B, an in vitro generated MHCII regulatory mutant (59).

RFXAP: The Regulatory Gene and Factor Affected in BLS Group D *RFXAP*, the gene encoding the second subunit of the RFX complex was identified using a biochemical approach (31). To isolate *RFXAP*, we purified the RFX complex, isolated its 36 kDa subunit, derived peptide sequences from this polypeptide, and then cloned the corresponding gene. The gene was called *RFXAP* (RFX Associated Protein) because it encodes a protein that interacts directly with RFX5 (31). The human *RFXAP* gene is situated on the long arm of chromosome 13 (55). The genomic structure of the mouse *Rfxap* gene has been determined and shown to consist of only 3 exons (W Reith, unpublished data). Defects in the *RFXAP* gene account for complementation group D (Figure 3). Three different mutations have been identified in six unrelated families (31, 55, 56, 86). An in vitro generated mutant (6.1.6) has also been shown to contain mutations in *RFXAP* (31).

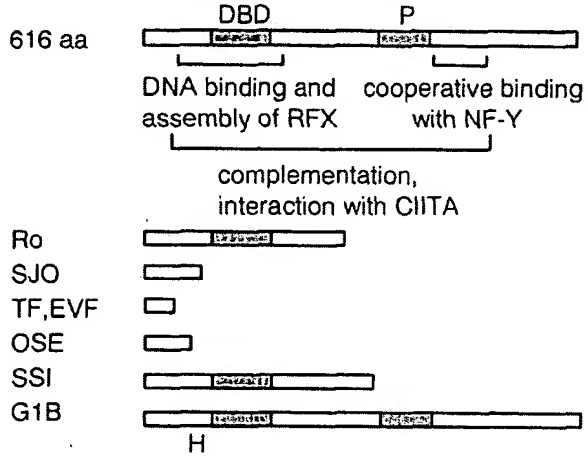
RFXANK: The Regulatory Gene and Factor Affected in BLS Group B *RFXANK* (also called *RFXB*), the gene encoding the last subunit of RFX, was also discovered thanks to biochemical approaches relying on purification of the RFX complex. It was identified on the basis of peptide sequences derived from the smallest 33 kDa subunit of RFX. We named the gene *RFXANK* because it encodes a factor containing a protein-protein interaction domain consisting of ankyrin repeats (Figure 3) (33). Subsequently, another group independently isolated the same gene and proposed the name RFXB (34). The human *RFXANK* gene consists of 10 exons spanning 9 kb of genomic DNA and is situated on the short arm of

Subunits of the RFX complex

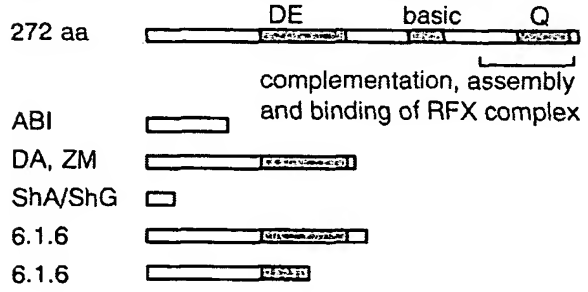
1) RFXANK (BLS complementation group B)



2) RFX5 (BLS complementation group C)



3) RFXAP (BLS complementation group D)



chromosome 19 (19p12) (33, 34). Defects in the *RFXANK* gene account for patients in complementation group B (33, 34, 87), which contains over 70% of all known BLS patients (Figure 3). Transfection of the *RFXANK* cDNA into cell lines from group B restores expression of all MHCII isotypes (33, 34). The *RFXANK* gene was found to be mutated in 26 unrelated patients (33, 34, 56, 87, 88). Only seven different mutations were identified in these patients (Figure 3). One of the mutations was found in 19 unrelated patients, indicating the existence of a founder effect (87).

Therapeutic Perspectives

Carrier Detection and Prenatal Diagnosis for BLS Prenatal diagnosis or carrier detection on a population-wide scale is not justified because BLS is a very rare disease. However, these procedures could be valuable for families in which patients have already been identified, or when a consanguineous union is envisaged in a high-risk population. Thanks to the identification of the molecular defects underlying BLS, mutated alleles of the *MHC2TA*, *RFXANK*, *RFX5*, and *RFXAP* genes can now be screened for directly. This will permit healthy carriers to be identified unambiguously and should allow the development of accurate and reliable procedures for prenatal diagnosis.

Gene Therapy for BLS Bone marrow transplantation (BMT) is currently the only curative treatment for BLS (89). Compared to other immunodeficiency syndromes, the success rate of BMT for BLS has been rather poor (89). This does not appear to be a peculiar characteristic of the BLS disease. Instead, it is likely to be due mainly to other criteria, such as diagnosis at a late age (89, 90). Identification of the affected genes has raised the hope that gene therapy will represent a potential alternative to BMT. Introduction of the wild-type MHC2TA, RFXANK, RFX5, or RFXAP genes into hematopoietic stem cells of BLS patients in complementation groups A, B, C, or D, respectively, would represent a logical therapeutic strategy. The *Mhc2ta* and *Rfx5* knockout mice (see below) will be invaluable for

Figure 3 Structure, function, and mutation of the RFX subunits that are affected in BLS complementation groups B, C, and D. Schematic maps of the RFXANK, RFX5, and RFXAP subunits are shown. The acidic region (DE) and ankyrin repeat domain of RFXANK, the DNA binding domain and proline rich segment of RFX5, and the acidic (DE), basic and glutamine-rich (Q) regions of RFXAP are represented as gray boxes. The regions that are required for complementation of BLS cells, for assembly and binding of the RFX complex, for cooperative binding between RFX and NF-Y, and for interaction with CIITA are indicated. Mutations in RFXANK, RFX5, and RFXAP have been characterized in BLS patients (Ra, BLS-1, EBA, FZA, B23, and B25 for RFXANK; Ro, SJO, TF/EVF, OSE, and SSI for RFX5; ABI, DA, ZM, and Sha/ShG for RFXAP) and in two in vitro generated cell lines (G1B and 6.1.6). The effects of the mutations at the level of the RFXANK, RFX5, and RFXAP proteins are represented schematically.

developing gene therapy procedures for BLS. Defective selection of $CD4^+$ T cells, resulting from the lack of MHCII expression on thymic epithelial cells, might be anticipated to represent an obstacle to gene therapy for BLS. However, the fact that classical BMT can cure BLS suggests that restoring MHCII expression on non–bone marrow–derived cells, such as thymic epithelial cells, is not absolutely essential. Another potential concern is that the therapeutic transgene could induce ectopic or abnormal levels of MHCII expression, which could have deleterious consequences and compromise the success of gene therapy. This will probably not be a major problem for RFX5, RFXANK (the largest group of patients), and RFXAP, which are expressed ubiquitously at nonlimiting levels in all cell types. On the other hand, this may represent a major dilemma in the case of CIITA because expression of the MHC2TA gene is tightly regulated.

Therapeutic Modulation of MHCII Expression MHCII expression is severely impaired in BLS patients. The same is true for most cell types in the mouse models of the disease (see below). This implies that no bypass or alternative pathway can compensate efficiently for a deficiency in RFX or CIITA. Moreover, although there are indications that the specificity of RFX and CIITA is not as strict as previously believed (see below), the expression of MHCII genes remains the major system in which these transcription factors are essential. Because of these features, inhibitors of CIITA, RFXANK, RFXAP, and RFX5 would be anticipated to have an efficient and selective effect on MHCII expression. CIITA, RFXANK, RFXAP, and RFX5 may thus represent prime targets for novel immunomodulatory drugs having wide applications in situations in which inhibition of MHCII expression might be desirable or beneficial, such as organ transplantation, autoimmune diseases, and inflammation in general.

The immunogenicity and rejection of tumors can be enhanced by activating MHCII expression, alone or in combination with costimulatory molecules (91–94). This has raised hopes that the introduction of CIITA into tumor cells to activate MHCII expression might enhance tumor immunogenicity and contribute to the success of tumor immunotherapy. Experiments designed to address the validity of this approach have been initiated (95).

Lessons from Mouse Models for BLS

Knockout mice that reproduce the molecular defects of BLS patients in groups A and C have been constructed by targeting of the *Mhc2ta* and *Rfx5* genes (96–99). Both of these models reproduce the major immunopathological characteristics of the human disease. There is a strong reduction of constitutive MHCII expression on professional APCs (B cells, DCs, and macrophages) and thymic epithelial cells. Induction of MHCII expression by IFNγ is also abolished. The loss of MHCII expression results in a severely compromised immune system.

The $CD4^+$ T cell population in *Mhc2ta* and *Rfx5* knockout mice is decreased at least tenfold (96–99). This strong reduction is a consequence of severely impaired

positive selection, which results from the loss of MHCII expression on epithelial cells in the thymus. Surprisingly, the reduction in $CD4^+$ T cells is considerably less pronounced in the human disease; $CD4^+$ T cell counts are rarely reduced greater than two- to threefold in BLS patients (1, 3). The reason for this discrepancy between the human and mouse phenotypes is not clear. One plausible explanation is that positive selection in the human disease is compromised only partially because low levels of residual MHCII expression are retained in the thymus. Whether or not this explanation is valid remains to be determined because MHCII expression patterns in the thymus have been examined only in a few isolated cases (100, 101). A second possibility is that the $CD4^+$ T cells in BLS patients have escaped the normal thymic selection processes. They could for example have been selected on ligands other than MHCII molecules. In MHCII deficient mice, a large proportion of the residual $CD4^+$ T cells are CD1-restricted (102). Such alternative selection pathways could be more prominent in BLS patients. Interestingly, an analysis of the T cell repertoire in BLS patients has revealed minor alterations, suggesting that the $CD4^+$ T cells in these patients may indeed have been subjected to unusual selection mechanisms (103, 104).

The *Mhc2ta−/−* and *Rfx5−/−* mice exhibit residual MHCII expression in certain cell types (Table 3). This implies that there are alternative pathways for MHCII expression that bypass partially the strict requirement for RFX5 and/or CIITA

TABLE 3 Cell surface MHC expression in *Rfx5−/−* and *Mhc2ta−/−* mice

Tissue/cell type	*Rfx5−/−*	*Mhc2ta−/−*
MHCII expression		
Thymic cortex	−	+ (Weak, patchy)
Thymic medulla	+ (Strong)	−
Naive B cells	−	−
IL4/LPS activated B cells	+ (Reduced 10 fold)	−
Germinal center B cells	Not determined	+ (Low level)
Macrophages − IFN-γ	−	−
Macrophages + IFN-γ	−	−
Splenic DCs	+ (15% of cells)	−
Bone marrow derived DCs	+ (5% of cells)	−
Lymph node DCs	Not determined	+ (Low level)
MHCI expression		
T cells	Reduced 10 fold	Normal
B cells	Reduced 2−5 fold	Normal
IFNγ induced expression	Normal	Normal

(96, 97, 99). The precise pattern of residual MHCII expression differs between *Mhc2ta−/−* and *Rfx5−/−* mice (Table 3). Residual MHCII expression in the *Mhc2ta−/−* mice concerns mainly a subset of thymic epithelial cells, dendritic cells in the paracortex of lymph nodes, and B cells in the germinal centers of the spleen and lymph nodes. In contrast, *Rfx5−/−* mice retain strong MHCII expression on dendritic cells in the thymic medulla and significant, albeit weaker, expression on a fraction of splenic and bone marrow–derived dendritic cells. Low MHCII expression is also induced on *Rfx5−/−* B cells activated with LPS and/or IL-4. This difference in the residual expression pattern is surprising because the human disease is phenotypically homogeneous. Although low expression has been observed in cells from certain BLS patients, no distinctive residual expression pattern discriminating between patients lacking RFX5 and CIITA has been described (1–3). This discrepancy between the human disease and the mouse models could reflect species-specific differences in the function of the two MHCII regulatory genes. However, it is also possible that phenotypic differences in residual MHCII expression exist in the human disease as well but have escaped attention because not enough patients have been examined in detail.

PART 2: MOLECULAR BASIS FOR THE REGULATION OF MHCII EXPRESSION

The RFX DNA-Binding Complex

Elucidation of the molecular defects underlying BLS complementation groups B, C, and D demonstrated that RFX is a multimeric complex consisting of RFXANK, RFX5, and RFXAP, three unrelated subunits sharing no sequence homology (Figure 3) (31–34). The stoichiometry of the three subunits within the RFX complex remains to be determined. Co-immunoprecipitation experiments performed with nuclear extracts, as well as with recombinant proteins, have indicated that the RFX complex is preassembled in solution prior to binding to DNA (33, 105, 106). All three subunits are essential for assembly of RFX (33, 105, 106). A deficiency in any one of the three subunits results in the inability to assemble a functional complex, which explains the absence of RFX binding activity in cells from complementation groups B, C, and D. It is unlikely that RFX contains additional unidentified subunits because a complex indistinguishable from the native protein can be assembled in vitro from recombinant RFX5, RFXAP, and RFXANK (105, 106).

All three RFX subunits can be cross-linked to the DNA within the X box *cis*-acting sequence (107). It remains unknown how RFXAP and RFXANK contribute to binding of RFX. On the other hand, RFX5 contains a well-known DNA binding domain (DBD), called the RFX domain (32, 81, 82). The RFX domain has been identified in a variety of DNA-binding proteins having diverse regulatory functions

in organisms ranging from yeast to human (81, 82, 108–110). The structure of the RFX domain of one member of the RFX family (RFX1) has recently been determined and shown to belong to the winged helix subfamily of the helix-turn-helix (HTH) proteins (111). Surprisingly, the RFX1 DBD binds DNA in a fashion radically different from that observed for all other known HTH proteins. Instead of relying on a recognition helix, the RFX1 DBD makes unprecedented use of a β-hairpin (called the wing) to recognize its binding site (111). The amino acids implicated in site-specific binding of the RFX1 DBD are strongly conserved in RFX5, implying that the latter interacts with its X box target site in a similar fashion (111).

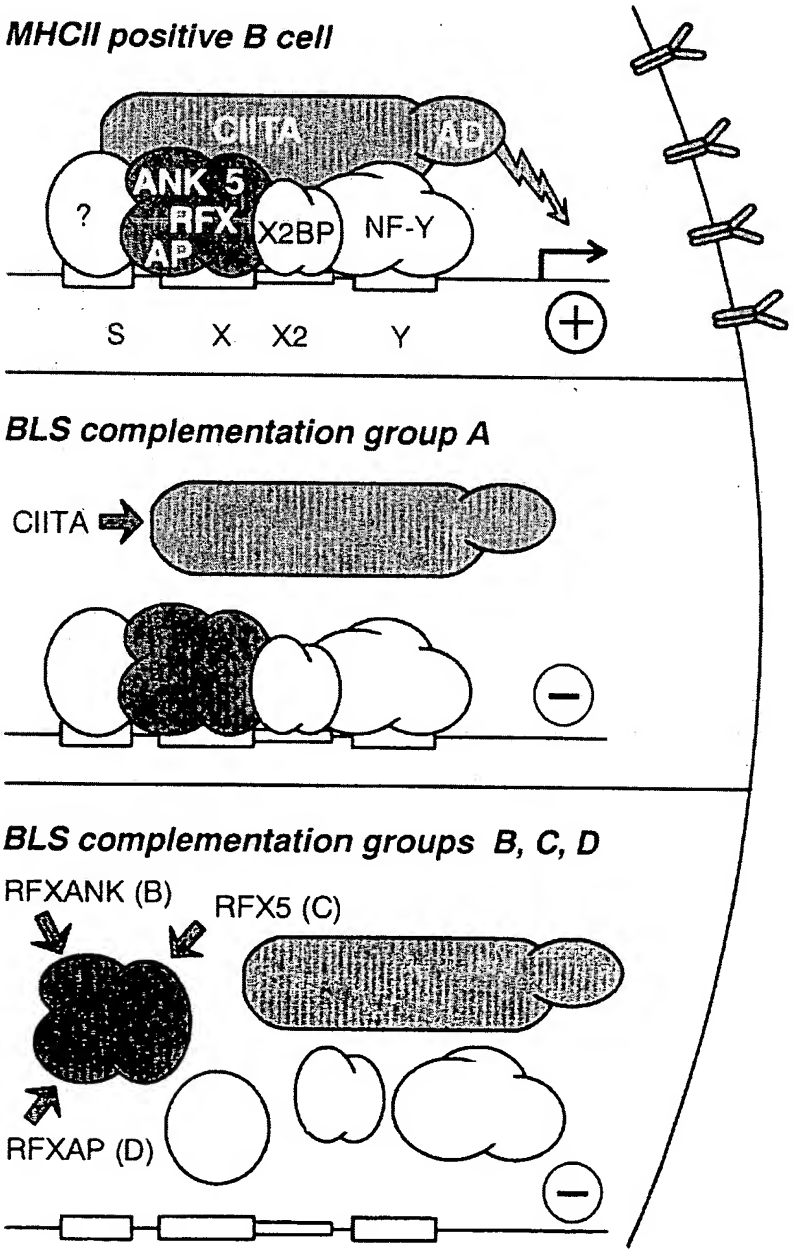
Functionally essential domains within RFXANK, RFX5, and RFXAP have been defined (Figure 3) (105, 106, 112, 113) (W Reith, unpublished data). The minimal essential region of RFXANK encompasses a C-terminal region containing a protein-protein interaction domain consisting of ankyrin repeats. This region is essential for assembly and binding of the RFX complex and for activation of MHCII promoters. All of the *RFXANK* mutations identified in BLS patients remove or affect the integrity of this minimal functional domain. The minimal region of RFX5 covers an internal segment containing the DBD and a proline rich region. This region is sufficient for assembly and binding of the RFX complex, cooperative binding with NF-Y (see below) and interaction with CIITA (see below). All of the *RFX5* mutations identified in BLS patients lead to deletion of the DBD and/or the proline rich region. In the case of RFXAP, the minimal region is restricted to a short C-terminal domain spanning a glutamine rich region. This region of RFXAP is sufficient for assembly and binding of the RFX complex and for activation of MHCII promoters. All of the mutations identified in BLS patients lead to the loss of this essential C-terminal region.

Several interesting features within the subunits of RFX appear to be dispensable for function (105, 106, 112, 113) (W Reith, unpublished data). RFXAP contains an N-terminal acidic region and a centrally placed basic region resembling a nuclear localization signal. Both of these regions can be removed without significantly affecting the function of RFXAP. The N-terminus of RFXANK contains an acidic region that has been suggested to be a PEST (proline/glutamic acid/serine/threonine) domain (34). PEST domains are frequently found in proteins that have short half-lives (114). Whether or not this region functions as a PEST domain remains to be determined. However, it is clearly not essential because it can be removed without eliminating the function of RFXANK.

RFX Promotes Assembly of an MHCII Enhanceosome Complex

One of the major roles of RFX is to promote stable binding of other transcription factors to MHCII promoters (Figure 5a). Strong evidence for this was first provided by DNAseI hypersensitivity studies and in vivo footprint experiments examining

the occupation of MHCII promoter in B cells that express or lack RFX (64–66). All of the critical *cis*-acting sequences of MHCII promoters are occupied by their cognate DNA-binding proteins in RFX-positive B cells (wild-type cells and cells from BLS complementation group A) (Figure 4). In RFX-deficient B cells (BLS complementation groups B-D), on the other hand, MHCII promoters are bare (Figure 4) (64–66). This defect in occupation is not restricted to the X box target site of RFX. Instead, the X2 and Y boxes are also unoccupied, indicating that



stable occupation of these sequences by their cognate DNA-binding factors is dependent on binding of RFX to the adjacent X box. Subsequent experiments explained this observation by demonstrating that RFX binds cooperatively (67–71, 115) with the Y box binding protein NF-Y (72) and the X2 box binding factor X2BP (67), which has recently been suggested to contain CREB (116) (Figure 5a). Cooperative binding interactions between these three proteins leads to the assembly of a remarkably stable higher order protein-DNA complex at MHCII promoters (67–71, 115).

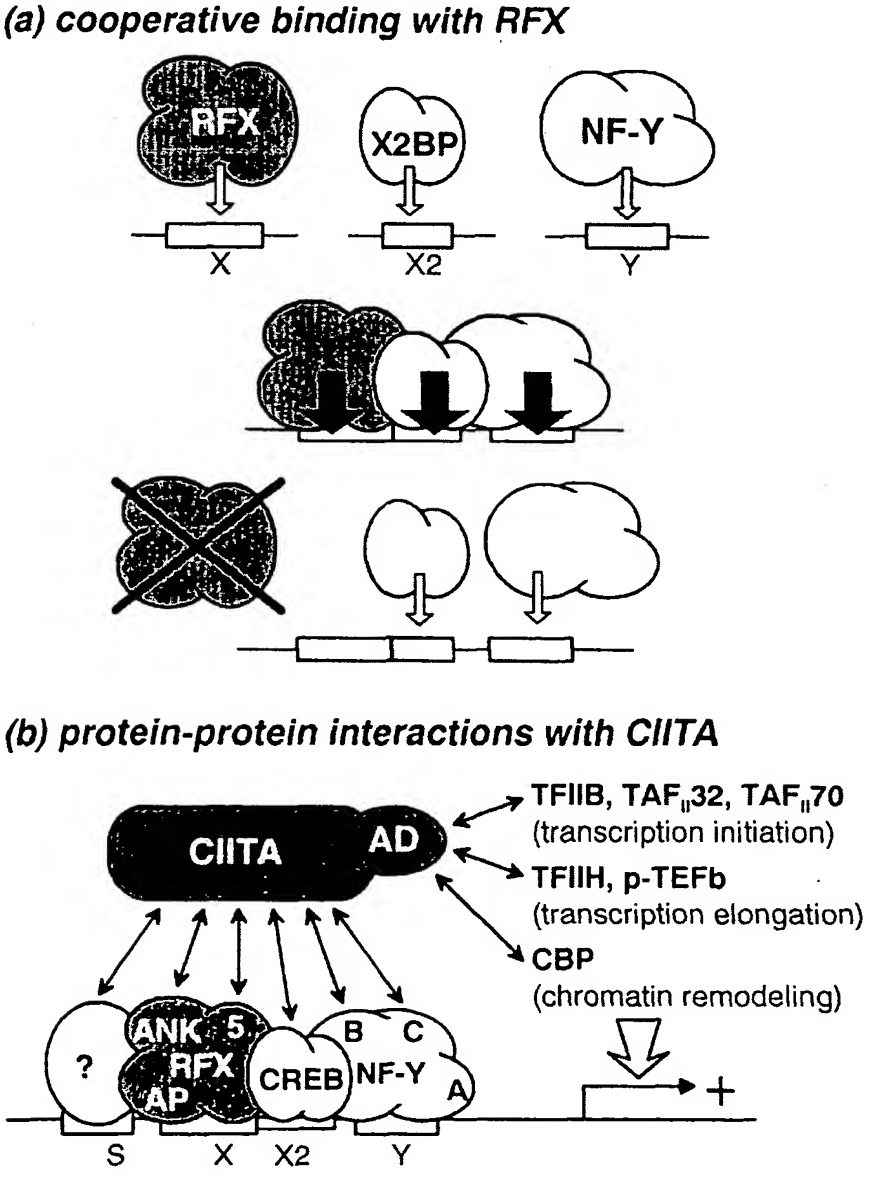
In addition to enhancing stability, the cooperative interactions are also specific, thereby ensuring that the X, X2, and Y boxes are bound in vivo by RFX, X2BP (CREB), and NF-Y rather than by the multitude of other factors capable of binding to these sequences with comparatively low affinity in vitro. It is of particular relevance here that several other members of the X box binding family of proteins can bind to the X box in vitro, but fail to interact with X2BP or NFY, and thus fail to stabilize the multiprotein complex on MHCII promoters in vivo. These other X box binding proteins (RFX1 to RFX4) control other genes, by binding to X box sequences flanked by protein-binding motifs distinct from those found in MHCII promoters (81, 82, 108–110)

The higher order nucleoprotein complex that assembles at MHCII promoters has recently been coined the MHCII 'enhanceosome' (115). The requirement for the assembly of this enhanceosome explains the observation that the S, X, X2, and Y sequences function together as a single composite regulatory module in which correct spacing and stereospecific alignment is critical (71, 117, 118). Protein domains mediating cooperative binding interactions within the MHCII enhanceosome have begun to be defined. A region situated immediately downstream of the proline-rich segment of RFX5 mediates cooperative binding between RFX and

Figure 4 Molecular defects in the bare lymphocyte syndrome. A typical MHCII promoter containing the conserved S, X, X2, and Y sequences is depicted. The in vivo promoter occupancy and transcription status (+ or −) are shown for wild-type MHCII positive B cells and for B cells from BLS patients in complementation groups A, B, C, and D. Key transcription factors controlling promoter activity are indicated. RFX, X2BP, and NF-Y bind cooperatively to the X, X2, and Y sequences to form a highly stable nucleoprotein complex referred to as the MHCII enhanceosome. Proteins binding to the S box (?) remain poorly characterized. CIITA is a non-DNA-binding coactivator that is recruited to the promoter via protein-protein interactions with the DNA-binding components of enhanceosome. Proteins binding to the S (?), X (RFX), X2 (X2BP), and Y (NF-Y) boxes are all required for CIITA recruitment. CIITA activates transcription via N-terminal transcription activation domains (AD). The gene encoding CIITA is mutated in BLS patients in complementation group A. Mutations in CIITA do not affect assembly of the enhanceosome, and promoter occupation is consequently not modified. The genes that are defective in complementation groups B, C, and D, encode, respectively, the RFXANK, RFX5, and RFXAP subunits of RFX. In contrast to defects in CIITA, mutations in RFXANK, RFX5, or RFXAP disrupt assembly of the enhanceosome and thus lead to a bare promoter.

NF-Y (105). At the level of NF-Y, the minimal evolutionarily conserved regions comprising the DNA-binding domain are sufficient for cooperative binding with RFX (106). The regions within RFX and X2BP (CREB) that are implicated in their cooperative binding have not yet been mapped.

In addition to the crucial role of the MHCII enhanceosome in enhancing the stability and specificity of promoter occupation, it has very recently also been shown that this enhanceosome constitutes a platform onto which the transcriptional coactivator CIITA is recruited via protein-protein interactions (Figures 4 and 5, see above) (115). Association of CIITA with the MHCII enhanceosome



is mediated by synergistic contacts with multiple components of the enhanceosome, including factors binding to the S, X, X2, and Y sequences (see below) (112, 113, 115, 119).

The Non-DNA-Binding Coactivator CIITA

The gene encoding CIITA was isolated by virtue of its ability to correct the genetic defect in cell lines from BLS complementation group A (29). Although this genetic approach provided irrefutable evidence that CIITA is a crucial transactivator of MHCII genes, it provided frustratingly little information on how it achieves this. A considerable amount of effort has consequently been devoted to the elucidation of the mode of action of CIITA.

Several clues concerning the mode of action of CIITA are embedded in the primary sequence of the protein, which exhibits four major features of interest (Figure 2). First, the N terminus of the protein contains a region rich in acidic amino acids. Second, downstream of this acidic region lie three segments rich in proline, serine, and threonine (PST). Third, there is a centrally placed GTP binding domain that contains three characteristic sequences, a Walker A motif (also known as a P-loop) involved in nucleotide binding, a magnesium binding site, and a sequence that is believed to confer GTP binding specificity. Finally, there is a leucine rich repeat (LRR)–based protein-protein interaction motif near the C terminus of the protein. All four features are conserved between human and mouse CIITA and are necessary for the ability of the protein to activate MHCII promoters (120–125).

The acidic and PST regions found within the N-terminal portion of CIITA resemble the transcription activation domains typically found in mammalian transcription factors. Several observations support the notion that these regions of

Figure 5 Mode of action of RFX and CIITA. (*a*) RFX participates in cooperative binding interactions required for promoter occupation in vivo. (*Top*) When bound individually, RFX, X2BP, and NF-Y have only low affinity (thin open arrows) for their respective X, X2, and Y box target sites. (*Middle*) Their binding affinity is strongly enhanced (thick black arrows) when the three proteins bind cooperatively to the same MHCII promoter fragment. (*Bottom*) In RFX-deficient BLS cells, cooperative binding is lost, and the residual low binding affinity of X2BP and NF-Y are not sufficient for stable promoter occupation in vivo. (*b*) CIITA is a non-DNA-binding transcriptional coactivator that functions at MHCII promoters via protein-protein interactions. Tethering of CIITA to MHCII and related promoters requires protein-protein interactions (arrows) with an as yet poorly defined S box binding protein, the RFXANK and RFX5 subunits of RFX, an X2 box binding protein (CREB), and the B and C subunits of NF-Y. Once tethered to the promoter, CIITA is believed to activate transcription by recruiting (arrows) other factors via its N-terminal activation domains (AD). Candidate factors recruited by CIITA include TFIIB, $TAF_{II}32$, $TAF_{II}70$, TFIIH, p-TEFb, and CBP. The putative effects of these factors on transcription initiation, elongation, and chromatin remodeling are indicated.

CIITA actually serve as bona fide transcription activation domains (Figure 2). First, they are essential for the ability of CIITA to activate MHCII promoters, and their deletion generates dominant negative mutants of CIITA (120, 121, 123, 124, 126, 127). Second, the acidic region can function as an activation domain when it is grafted onto a heterologous DNA-binding protein such as GAL4 (124). Finally, the function of N-terminally deleted versions of CIITA can be partially rescued by fusing them to the activation domains of the transcription factors VP16 and TIF (124, 125).

At least three different mechanisms have been put forward to explain how the activation domains of CIITA stimulate transcription (Figure 5b). First, CIITA can interact with the general transcription factors TFIIB, $hTAF_{II}32$, and $hTAF_{II}70$, suggesting that it promotes transcription initiation by recruiting the general transcription machinery (128, 129). Second, CIITA has been proposed to enhance promoter clearance or transcription elongation by interacting with TFIIH or P-TEFb (129, 130). Third, CIITA may facilitate chromatin remodeling by recruiting the histone acetyltransferase CBP (131, 132). The latter mechanism could potentially explain why the accessibility of MHCII promoters to DNA-binding proteins is enhanced by CIITA in certain cell types (133, 134). These three mechanisms are not mutually exclusive, and CIITA could well function as a scaffold for the recruitment of several factors that activate transcription at different levels.

Very early on, it was recognized that CIITA does not activate transcription of MHCII genes by binding directly to the promoter DNA. This suggested that CIITA is a coactivator recruited to MHCII promoters by means of protein-protein interactions with one or more of the factors binding to the S, X, X2, and Y sequences (Figure 5b). Definitive evidence for this hypothesis has been provided only very recently (112, 113, 115, 119). Chromatin immunoprecipitation experiments have revealed that CIITA is indeed physically associated in vivo with MHCII promoters (115), both several different MHCII promoters (*HLA-DRA*, *HLA-DRB1*, and *HLA-DPB*) and the promoters of the *Ii* and *HLA-DM* genes (115). Furthermore, in vitro recruitment experiments have demonstrated that tethering of CIITA to MHCII promoters is mediated by multiple synergistic protein-protein interactions with components of the MHCII enhanceosome (Figure 5b). Factors bound to the S, X, X2, and Y boxes are all required for recruitment of CIITA (115). Yeast two-hybrid and coimmunoprecipitation experiments have shown that CIITA interacts directly with the RFXANK and RFX5 subunits of RFX, the B and C subunits of NF-Y, and CREB (Figure 5b) (112, 113, 119, 135).

Domains within CIITA that interact with the DNA bound factors have begun to be defined. It is clear that recruitment to the MHCII enhanceosome requires the C-terminal moiety of CIITA (Figure 2) (115). This is consistent with the earlier observation that the specificity of CIITA for MHCII promoters is conferred by its C-terminal portion (125). Moreover, domains mediating interactions with RFX5 and NF-YB have been mapped within this C-terminal region of CIITA (113).

The LRR region is also important because mutations disrupting this region in BLS patients reduce the CIITA-enhanceosome interaction (115). Results concerning the importance of the N-terminal portion of CIITA for recruitment are less coherent. A recent study has defined the N terminus of CIITA as being the region that mediates interactions with RFXANK and NF-YC (113). This is at odds with the observation that this region is actually dispensable for recruitment (115). In fact, dominant negative mutants of CIITA lacking the N-terminal activation domains actually bind better to the MHCII enhanceosome, suggesting that these dominant negative mutants are likely to function by blocking access of wild-type CIITA to the promoter (115). Further work will be required to reconcile these contradictory findings.

Three regions within CIITA have been implicated in nuclear localization (Figure 2). First, the C terminus of CIITA contains a 5-amino-acid motif resembling a nuclear localization signal (NLS) (136). This NLS motif is essential for nuclear localization of CIITA and can direct nuclear import in a manner that is independent of other CIITA sequences. A 24-amino-acid deletion removing this NLS in the BLS patient BLS-2 aborts nuclear import of CIITA (136). A second sequence implicated in nuclear import is the GTP-binding motif. Binding of GTP appears to be required for transport of CIITA into the nucleus (137). Finally, a detailed mutational analysis of the LRR region has shown that it is important for directing CIITA to the nucleus (119). It remains to be determined whether these three regions are redundant and function independently of each other, or if they act together in a concerted fashion. Whether the nuclear-cytoplasmic distribution of CIITA is regulated also remains an open question.

CIITA shares a similar architecture and a low level of sequence homology with a set of other proteins. All of these proteins contain a nucleotide-binding domain (NBD) coupled to a LRR domain situated near the C terminus (138, 139). These other proteins have functions very different from that of CIITA. They include the caspase recruitment protein Nod1 and certain plant disease resistance proteins (138, 139). Conservation of the NBD-LRR domain organization in proteins having very different functions is intriguing. It is tempting to speculate that the similar structure of these proteins reflects an as yet unknown analogy in their mode of action.

CIITA Is the Master Regulator of MHCII Expression

In contrast to the ubiquitous expression of the DNA-binding factors (including RFX) constituting the MHCII enhanceosome, expression of the *MHC2TA* gene is tightly regulated. In the majority of situations it is the expression of CIITA that dictates whether, and at what level, MHCII genes are expressed. The *MHC2TA* gene is therefore the master regulator of MHCII expression and hence has an essential immunomodulatory role. This conclusion has been firmly established by the following findings. First, the analysis of a large number of human and mouse cell lines

and tissues has demonstrated that there is a strict correlation between MHCII and *MHC2TA* expression. Second, the majority of cell types do not express *MHC2TA* and are consequently MHCII-negative. Expression of the *MHC2TA* gene, and thus of MHCII, can be activated in such cells by stimulation with IFNγ (140, 141). This has been demonstrated in a variety of established cell lines, including fibroblasts, melanoma cells, and macrophages, and in several primary cell types such as mouse embryonic fibroblasts, peritoneal macrophages, microglia, and astrocytes (140–148). Transfection of MHCII-negative cells with CIITA expression vectors is generally sufficient to induce MHCII expression (140, 141, 149, 150). The latter point in particular emphasizes the fact that CIITA is both essential and sufficient, thereby justifying the notion that it represents the master regulator of MHCII expression. Third, experimental modulation of *MHC2TA* expression using a tetracycline inducible system has shown that the level of *MHC2TA* expression directly determines that of MHCII expression (151). Fourth, constitutive expression of MHCII in B cells and dendritic cells is sustained by constitutive expression of CIITA (29). Fifth, extinction of MHCII expression during the differentiation of B cells into mature plasma cells is caused by silencing of the *MHC2TA* gene (149, 152). Sixth, MHCII expression is induced upon activation of human T cells because the *MHC2TA* gene is switched on (A Muhlethaler-Mottet, B Mach, unpublished data). Activation of mouse T cells on the other hand does not turn on the *MHC2TA* gene, and hence MHCII expression is not induced (153). Seventh, repression of MHCII expression in trophoblast cells is caused by inhibition of *MHC2TA* expression (154, 155). Finally, an important emerging concept is that a variety of pathogens have developed the ability to inhibit CIITA expression—and thus MHCII expression—as a strategy to evade recognition by the immune system. Examples include cytomegalovirus (CMV) (156, 157), varicella-zoster virus (VZV) (158), *Mycobacterium bovis* (159) and chlamydia (160). This last point underlines the functional importance of CIITA as an essential immunomodulator.

Although the level of transcription of the *MHC2TA* gene is in the majority of situations the dominant factor dictating MHCII expression, other levels of regulation have also been reported. The modulatory effects of IFNβ and TNFα on IFNγ–induced MHCII expression act downstream of *MHC2TA* gene transcription (161–163). Moreover, weak or isotype specific MHCII expression has been observed in the absence of CIITA in certain mutant human cell lines (164) and in certain cell types in CIITA-deficient mice (Table 3) (96, 97). However, these examples of CIITA-independent MHCII expression remain exceptions, and the mechanisms involved are not yet understood.

Regulation of CIITA Expression

The fact that the expression pattern of MHCII genes is determined primarily by that of CIITA has motivated substantial interest in the mechanisms controlling transcription of the *MHC2TA* gene. A large (greater than 12 kb) and complex

regulatory region containing several independent promoters controls transcription of *MHC2TA* (Figure 6a). Four promoters (pI, pII, pIII, and pIV) have been identified in the human gene, three of which (pI, pIII, and pIV) are also strongly conserved in the mouse gene (145). The usage of these promoters leads to the synthesis of distinct CIITA mRNAs (types I, III, and IV) containing alternative first exons spliced to a shared second exon (Figure 6a) (145). The study of the different *MHC2TA* promoters has demonstrated that it is their differential activity that ultimately determines the complex expression pattern of MHCII genes that is observed in vivo (145). This has been confirmed in several biological systems (144–147, 162, 165–167). Different cellular and functional specificities of MHCII expression have thus been allocated to distinct CIITA promoters, each one having a specific physiological relevance (Figure 6a).

In both humans and mice, pI is highly specific for DCs. Type I CIITA mRNA typically represents a preponderant fraction of the total CIITA mRNA found in various different DC preparations (145, 165). This is for instance the case for splenic and thymic DCs isolated ex vivo from the mouse, DCs derived from mouse bone marrow cultures and human monocyte–derived DCs (145, 165; W Reith, B Mach, unpublished data). However, pI is not the only promoter active in DCs; there is generally also a variable but significant fraction of type III CIITA mRNA (145; W Reith, unpublished data). Moreover, DC specificity of pI is not absolute; it is also used in mouse microglia and peritoneal macrophages following activation with IFNγ (165) (JM Waldburger, W Reith, manuscript in preparation). Type I transcripts contain an alternative first exon that contains a translation initiation codon and codes for a specific 94-amino-acid N-terminal extension of CIITA (Figure 6a) (145). CIITA protein isoforms containing this type I specific extension are detected in DCs (W Reith, B Mach, unpublished data).

PIII is used primarily in B cells (145, 147, 166, 167). Sequences important for B cell specificity of this promoter have been defined (166). It should however be mentioned that the B cell specificity of pIII is not absolute. It is for example also used in certain DC preparations (145; W Reith, unpublished data). An IFNγ–responsive enhancer has moreover been mapped upstream of pIII in the human gene (146). Type III CIITA mRNA contains an alternative first exon that contains a translation initiation codon and encodes a specific 17-amino-acid N-terminal extension (Figure 6a) (145). CIITA protein isoforms containing this type III specific extension are detected in B cells (W Reith, B Mach, unpublished data).

PIV is activated by IFNγ in a wide variety of established cell lines and primary cell types, including monocytes, macrophages, microglia, astrocytes, fibroblasts, and endothelial cells (143–147, 168). The function of pIV has been dissected in considerable detail, which has permitted the complete signal transduction pathway from the IFNγ receptor to MHCII expression to be elucidated (Figure 6b) (143–147, 168). IFNγ–induced activation of pIV relies on three *cis*-acting sequences (a GAS element, an IRF binding site, and an E box) that function together

in synergy. The GAS and IRF sequences are bound, respectively, by STAT1 and IRF1 (144), two transcription factors that mediate IFNγ–induced gene expression in a variety of other systems (169–171). The relative contribution of STAT1 and IRF1 for pIV activation varies according to the cell type examined (146, 162). The E box is bound by USF1, a ubiquitously expressed transcription factor that binds cooperatively with STAT1 (144). Unlike the type I and III mRNAs, the type IV–specific exon does not contain a translation initiation codon and does therefore not encode an N-terminal extension (Figure 6a) (145). Translation of type IV CIITA mRNA is initiated at an initiation codon situated in the shared second exon (145).

To further refine the analysis of pIV usage we have excised it by gene targeting in mice (JM Waldburger, W Reith, manuscript in preparation). As expected, IFNγ–induced MHCII expression in pIV−/− mice is abolished in non–bone marrow–derived cells, including fibroblasts, astrocytes, hepatocytes, endothelial cells, and epithelial cells. Surprisingly, however, the excision of pIV does not eliminate MHCII expression in peritoneal macrophages and microglial cells following their activation with IFNγ. Thus, while pIV is essential for IFNγ–induced MHCII expression in non–bone marrow–derived cells, it is dispensable for MHCII expression in IFNγ–activated cells of the macrophage lineage. Enhanced MHCII expression in IFNγ–activated pIV−/− macrophages is due to the fact that pI is turned on in these cells. A second unexpected finding is that pIV−/− mice completely lack CIITA expression on epithelial cells of the thymic cortex. Since MHCII expression on these cells is essential for positive selection of $CD4^+$ thymocytes (7), pIV−/− mice have very low $CD4^+$ T cell counts.

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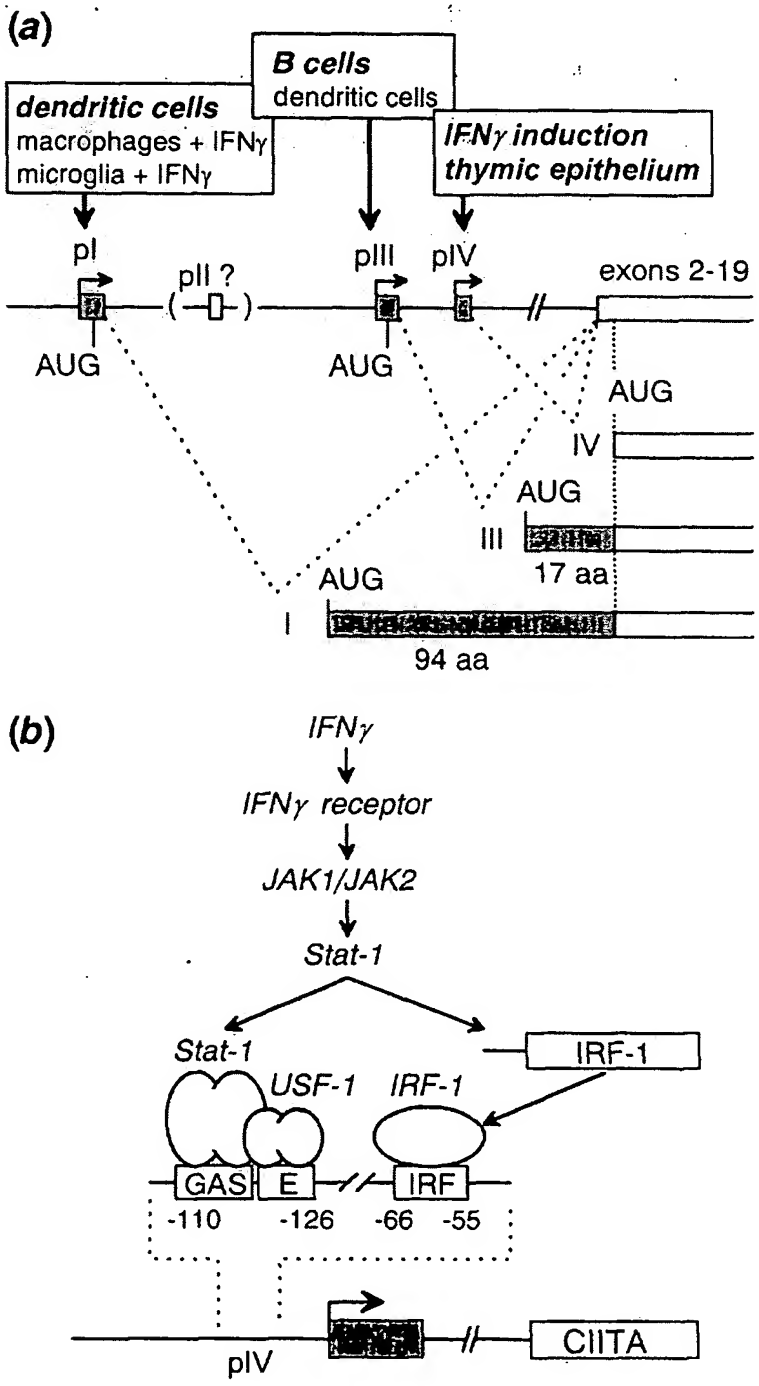
Figure 6 Expression of the *MHC2TA* gene. (*a*) Several independent promoters drive transcription of the *MHC2TA* gene. Four promoters (pI, pII, pIII, pIV) have been identified in humans. Only three of these (pI, pIII, pIV) are conserved in the mouse. Usage of these promoters leads to the splicing (dashed lines) of alternative first exons to a shared second exon. Incorporation of exons I and III lead to the synthesis of type I and type III CIITA protein isoforms having specific N-terminal extensions of 94 and 17 amino acids, respectively. Translation of type IV CIITA is initiated at the AUG found in the shared second exon. The promoters exhibit different activities. pI is used primarily in human and mouse dendritic cells. It is also active in activated mouse macrophages and microglia. pIII is used mainly in B cells, but also to a variable extent in dendritic cells. pIV is essential for IFNγ–induced expression in non–bone marrow–derived cells, as well as for expression in thymic epithelial cells. (*b*) The entire signal transduction cascade mediating the induction of MHCII expression by IFNγ has been elucidated. Activation of Jak1 and Jak2 kinases by interaction of IFNγ with its receptor leads to the phosphorylation, dimerization, and nuclear import of STAT1. STAT1 binds cooperatively with USF-1 to a composite GAS/E box motif present in pIV of the *MHC2TA* gene. STAT1 also activates expression of IRF-1, which then binds to its cognate site in pIV. Activation of pIV by STAT1, USF-1, and IRF-1 leads to expression of CIITA, and thus to the induction of MHCII expression.



Induction of MHCII Expression: A Pathway Affected in Multiple Situations

pIV of the *MHC2TA* gene is turning out to be a privileged focal point for a growing number of influences that inhibit or abort MHCII expression, both under normal physiological situations and during the course of disease. First, a variety of cytokines known to inhibit IFNγ–induced MHCII expression, including TGFβ, L-4, IL-10, and IL-1β, have been shown to mediate their inhibitory effect by interfering with the induction of *MHC2TA* expression (142, 147, 168, 172, 173). Second, in trophoblast cells (154, 155) and in certain IFNγ unresponsive tumors (174, 175), the *MHC2TA* gene is refractory to induction by IFNγ. Third, the production of NO by activated macrophages inhibits IFNγ–induced activation of CIITA expression (176). Fourth, a number of pathogens, including cytomegalovirus (CMV) (156, 157), varicella-zoster virus (VZV) (158), *Mycobacterium bovis* (159) and Chlamydia (160) exhibit the ability to inhibit MHCII expression by interfering with the activation of CIITA expression. Finally, an emerging concept is that certain drugs can interfere with activation of MHCII expression. For instance, it has been shown very recently that the lipid lowering drugs statins inhibit IFNγ–induced CIITA expression (177). These various inhibitory effects likely converge on pIV of the *CIITA* gene, although evidence in support of this has been provided only for some of them (trophoblast cells, IL-1, TGFβ, CMV, Chlamydia, and statins) (142, 146, 147, 155–157, 160, 168, 172, 173).

So far, the molecular mechanisms underlying the inhibitory effect on activation of pIV by IFNγ have only been addressed in certain cases. In trophoblast cells, the lack of IFNγ inducibility of CIITA is due to methylation of pIV (155). The inhibition by CMV has been suggested to be due to enhanced proteasome-mediated degradation of Jak1, thereby limiting activation of Stat1 in response to IFNγ (156). Similarly, infection with VZV is associated with reduced levels of Stat1, Jak2, and IRF1, all of which would be expected to reduce induction of pIV (158). Finally, Chlamydia induces proteasome-mediated degradation of USF-1 (160), a ubiquitous transcription factor required for the activation of pIV (144). In addition to a reduction in the availability or activity of Stat1, USF1, or IRF1, other putative mechanisms could also be envisaged. A plausible target would, for example, be the cooperative binding interaction that takes place at pIV between Stat1 and IRF1 (144).

Specificity of RFX and CIITA

The clinical and immunological phenotypes of BLS patients in all four complementation groups can be explained by the absence of antigen presentation via MHCII molecules. This has been interpreted to indicate that RFX and CIITA are highly specific for the expression of the MHCII genes (4). The finding that RFX and CIITA are also required for expression of the genes encoding Ii, HLA-DM, and HLA-DO (14–19, 47) did not really challenge this specificity because

these proteins are involved in the intercellular traffic and peptide loading of MHCII molecules. However, recent evidence has implicated RFX and CIITA in three other unrelated systems, demonstrating that their specificity for the MHCII system is not as tight as initially believed.

In addition to the profound deficiency in MHCII expression, a reduction in MHCI and $\beta 2$ microglobulin is observed on various cell types from many BLS patients (Table 1) (1). Moreover, a reduction in MHCI expression has also been observed in *Rfx5−/−* mice; cell surface levels of MHCI in *Rfx5−/−* mice are lowered tenfold in T cells and two- to fivefold in B cells (W Reith, unpublished data). Taken together, these observations suggest that RFX and CIITA contribute to the expression of MHCI genes. This has been confirmed directly by several studies (23, 25–27) (W Reith, unpublished data). First, it has been established that *MHCI* and *B2M* promoters contain a region showing homology to the MHCII regulatory module consisting of the S, X, X2, and Y sequences (23–27). Second, RFX and CIITA can transactivate *MHCI* and *B2M* promoters in transient transfection experiments (23, 25–27, 178, 179) (W Reith, unpublished data). Third, certain BLS cell lines show enhanced levels of MHCI expression once their genetic defect has been corrected (26). Finally, chromatin immunoprecipitation experiments have demonstrated that CIITA and RFX are physically associated with MHCI and *B2M* promoters in vivo (115).

The existence of an additional gene affected by CIITA has been revealed by the analysis of *Mhc2ta* knockout mice. Expression of IL-4 is the hallmark of Th2 cells, whereas this cytokine is normally not expressed by Th1 cells (180). Disruption of the *Mhc2ta* gene has been reported to lead to the aberrant activation of IL-4 expression in Th1 cells (181), suggesting that CIITA is required for repression of the *IL-4* gene in these cells. This is apparently not due to a direct effect of CIITA on the *IL-4* promoter. Instead, CIITA expressed in Th1 cells has been proposed to bind to and sequester the coactivator CBP, which is required for transcription of the *IL-4* gene (182).

An interesting role of CIITA in HIV infection has recently been documented (130, 183). Expression of CIITA in T cells enhances HIV-1 replication and transcriptional activity of the HIV-LTR (183). This finding is of particular interest because the primary cellular targets of HIV—activated human T cells and macrophages—express high levels of CIITA. The mechanism by which CIITA activates the HIV-LTR remains obscure.

The MHCII System as a Textbook Model for Regulation of Gene Expression

Thanks to elucidation of the BLS disease, the regulation of MHCII gene expression is one of the only mammalian transcriptional control systems that has been dissected genetically in detail (Figure 4). Moreover, several features make the regulation of MHCII genes a textbook model system for the regulation of gene expression. First, it represents an unprecedented paradigm for the role of gene-specific

coactivators such as CIITA (Figure 5b). Second, it is among the few examples in which strong evidence for the involvement of a higher order enhanceosome complex has been provided (Figure 4 and 5A). Third, the MHCII system makes a very strong case for the key importance of combinatorial control mediated by cooperative protein-protein interactions. Such cooperative interactions are crucial at three levels in the MHCII system. At the MHCII promoter, the stability and specificity of enhanceosome assembly is strictly dependent on cooperative binding between at least three different DNA-binding factors, RFX, X2BP, and NF-Y (Figure 5a). The specificity of the coactivator CIITA for MHCII promoters is mediated by the combined effect of multiple weak protein-protein interactions with at least six different components of the DNA-bound enhanceosome complex (Figure 5b). Finally, the IFNγ–induced expression of CIITA is dependent on the cooperative binding of Stat1 and USF1 to a composite response element in pIV of the *MHC2TA* gene (Figure 6b). Taken together, these features make the MHCII system a valuable blueprint of widespread interest to the field of regulation of gene expression.

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Mini-review:

Specificity and expression of CIITA, the master regulator of MHC class II genes

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The class II transactivator (CIITA) has been referred to as the "master control factor" for the expression of MHC class II (MHCII) genes. As our knowledge on the specificity and function of CIITA grows, it is becoming increasingly evident that this sobriquet is entirely justified. First, despite extensive investigations, the major target genes of CIITA remain those implicated in the presentation of antigenic peptides by MHCII molecules. Although other putative target genes have been reported, the contribution of CIITA to their expression remains indirect, controversial or comparatively minor relative to its decisive role as a regulator of MHCII and related genes. Second, the most important parameter dictating MHCII expression is by far the expression pattern of the gene encoding CIITA (*MHC2TA*). The vast majority of signals that activate or repress MHCII expression under physiological and pathological situations converge on one or more of the three alternative promoters that drive transcription of the *MHC2TA* gene. In short, with respect to its specificity and its exquisitely controlled pattern of expression, CIITA is by a long stretch the single most important transcription factor for the regulation of genes required for MHCII-restricted antigen-presentation.

Key words: MHC class II / Class II transactivator / Gene expression / Repression / Cell type specificity

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1 Introduction

MHC class II (MHCII) molecules play a pivotal role in the induction and regulation of adaptive immune responses to pathogens. They are also central to the maintenance of self-tolerance and to the breaking of this tolerance during the initiation and development of autoimmune diseases. MHCII molecules are displayed at the surface of APC where they present peptides to the TCR of $CD4^+$ T cells. This triggers the activation and proliferation of the T cells and thus elicits an immune response directed against the antigen from which the MHCII-bound peptides were derived. MHCII molecules are also crucial for selection and maturation of $CD4^+$ T cells in the thymus. Positive selection, which ensures the survival of T cells that carry TCR capable of recognizing self-MHC molecules, is believed to be driven by $MHCII^+$ cortical thymic epithelial cells (cTEC) [1]. On the other hand, elimination of autoreactive T cells by negative selection is driven by $MHCII^+$ thymic DC and/or medullary thymic epithelial cells (mTEC) [1–3].

Constitutive expression of MHCII molecules is largely restricted to APC, namely dendritic cells (DC), B cells and macrophages. In addition, thymic epithelial cells (TEC) and activated human T cells express MHCII. In many $MHCII^-$ cell types, MHCII expression can be induced by various stimuli of which IFN-γ is by far the most potent. Both constitutive and induced MHCII expression can be further modulated by additional signals. Constitutive expression in B cells and DC is for instance regulated as a function of developmental stage and can be modulated by various cytokines. IFN-γ-induced expression can also be inhibited by numerous stimuli such as TGF-β, IFN-α and IL-4.

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Abbreviations: CIITA: Class II transactivator **cTEC:** Cortical TEC **LTR:** Long terminal repeat **MHCII:** MHC class II **mTEC:** Medullary TEC **pDC:** Plasmacytoid DC **RFX:** Regulatory factor X **SOCS-1:** Suppressor of cytokine signaling-1 **TEC:** Thymic epithelial cell **USF-1:** Upstream regulatory factor-1

MHCII expression is regulated mainly at the level of transcription [4, 5]. The promoters of MHCII and related genes are characterized by the presence of conserved sequence elements referred to as the W (or S), X, X2 and Y boxes (Fig. 1). The X box is bound by RFX (regulatory factor X), a trimeric complex composed of RFX5 (a member of the RFX family of DNA-binding proteins), RFXANK (also called RFX-B) and RFXAP [6–9]. The X2 box is recognized by X2BP, a complex that includes CREB [10]. Finally, the trimeric NF-Y complex, composed of NF-YA, NF-YB and NF-YC, binds to the Y box [11]. A number of proteins can bind the W box *in vitro*, including RFX [12], but none of them has been formally shown to be the functionally relevant W-box-binding protein *in vivo*.

All of these factors binding to the *cis*-regulatory elements of MHCII promoters are required for MHCII gene expression. They bind cooperatively to the promoter to form a highly stable macromolecular nucleoprotein complex referred to as the MHCII enhanceosome [13] (Fig. 1). The enhanceosome serves as a landing pad for the class II transactivator (CIITA) [13, 14]. CIITA is a non-DNA-binding coactivator that serves as the master control factor for MHCII expression [4, 5].

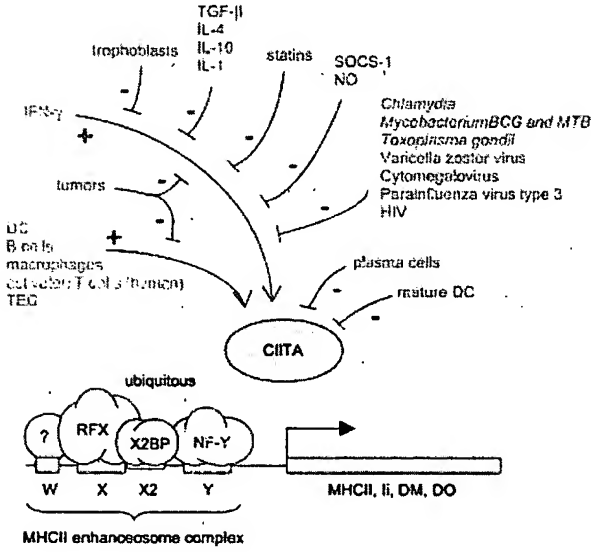

Fig. 1. Activation and silencing of the *MHC2TA* gene. Positive and negative influences on CIITA expression are indicated in green and red, respectively. DC, B cells, macrophages, activated human T cells and TEC express CIITA constitutively. The expression of CIITA is repressed during terminal differentiation of B cells into plasma cells and of immature DC into mature DC. CIITA expression is induced by IFN-γ in most other cell types and this is repressed by many factors including cytokines (TGF-β, IL-4, IL-10 and IL-1), statins, negative feedback regulators (SOCS-1 and NO) and a growing number of pathogens, including *Chlamydia*, *Mycobacterium* BCG and MTB, *Toxoplasma*, varicella zoster virus, cytomegalovirus, parainfluenza virus type 3 and HIV. IFN-γ-induced CIITA expression is also repressed in fetal trophoblasts. Both constitutive and IFN-γ-induced CIITA expression are frequently silenced in tumor cell lines of various origins.

The enhanceosome components are expressed more or less ubiquitously and thus fail to account for either the cell-type-specificity or IFN-γ-inducibility of MHCII expression. In contrast, CIITA exhibits a cell-type-specific, cytokine-inducible and differentiation-stage-specific pattern of expression that precisely parallels that of MHCII genes [4, 5] (Fig. 1). Thus, MHCII$^+$ cells such as DC, B cells, macrophages and TEC express CIITA. In addition, CIITA expression is up-regulated by IFN-γ in most other cell types and this induced expression is down-regulated by numerous other stimuli. In all of these situations it has been firmly established that it is indeed the expression of CIITA that is responsible for driving the activation of MHCII genes. The regulation of CIITA expression occurs primarily at the level of transcription of the *MHC2TA* gene.

Numerous original articles have addressed the structure and mode of action of CIITA and this subject has been discussed in recent reviews [4, 5, 15, 16]. Here we will instead concentrate on the specificity of CIITA, the activation of its expression in different cell types and its silencing under physiological and pathological conditions.

2 The specificity of CIITA

MHCII and related genes are undoubtedly the most important target genes of CIITA. In addition to the genes encoding classical MHCII molecules (HLA-DR, -DP and -DQ), CIITA activates the expression of several genes encoding accessory proteins required for MHCII-restricted antigen-presentation, namely the invariant chain (Ii), HLA-DM and HLA-DO [17–20]. It also contributes, albeit to a lesser degree, to classical and non-classical MHCI expression [4, 16, 21, 22]. The expression of multiple genes involved in antigen presentation is thus controlled either completely or in part by CIITA. This clearly remains the primary function of CIITA. However, a series of recent papers have suggested that CIITA may also be implicated in other functions within and outside the immune system (Table 1).

IL-4, a Th2-type cytokine, was found to be aberrantly up-regulated in Th1 cells derived from CIITA knockout mice [23]. In addition, CIITA was reported to be expressed in wild-type CD4$^+$ T cell preparations under Th1 but not Th2

Table 1. Previously known and novel targets of CIITA

Target gene	Function	Expression	Comments	Effect of CIITA	Fold effect[a]	Mechanism	Ref.
HLA-DR HLA-DP HLA-DQ	Classical MHCII molecules, presentation of peptides to TCR of CD4+ T cells	APC, TEC, IFN-γ-induced cells	Expression abolished in CIITA-deficient bare lymphocyte syndrome (BLS) patients in complementation group A	Activation	>1000 x[b]	Activation by binding to the MHCII enhanceosome	[14]
HLA-DM HLA-DO Ii chain	Non-classical MHCII molecules, accessory proteins required for MHCII-restricted antigen presentation	APC, TEC, IFN-γ-induced cells	Expression reduced in CIITA-deficient BLS patients in complementation group A	Activation	10 x[c]	Activation by binding to the MHCII enhanceosome	[17–20]
MHCI	Presentation of peptides to TCR of CD8+ T cells	Ubiquitous	MHCI promoters contain W–X–X2–Y motif, expression reduced in BLS patients	Activation	5–8 x[d]	Activation by binding to the MHCII enhanceosome	[21, 22]
IL-4	Th2 cytokine	Th2 cells	IL-4 expression increased in CIITA knockout mice	Repression	6 x[e]	Sequestration of CBP	[23, 25]
FasL	Mediator of apoptosis	T cells, immune-privileged sites	FasL expression increased in CIITA knockout mice	Repression	5 x[f]	Sequestration of CBP	[24, 27]
Collagen α2(I)	Extracellular matrix protein	Fibroblasts osteoblasts odontoblasts	Collagen α2 (I) promoter activity inhibited in CIITA$^+$ cells	Repression	5 x[g]	Sequestration of CBP	[28]
HIV (LTR)	Proviral promoter	HIV-infected cells	HIV transcription enhanced in CIITA$^+$ cells	Activation	6 x[h]	Unknown	[30, 31]
HIV (LTR)	Proviral promoter	HIV-infected cells	HIV transcription decreased in CIITA$^+$ cells	Repression	5 x[i]	Inhibition of viral transactivator Tat	[33, 34]
Plexin-A1	Semaphorin receptor, contributes to DC-mediated T cell stimulation	Mature DC	Reduction of Plexin-A1 expression in DC from CIITA knockout mice	Activation	20 x[j]	Unknown	[35]
Others	Various functions	Various	Microarray analysis comparing wild-type and CIITA-deficient B cells	Activation or repression	2–10 x[k]	Unknown	[37]

[a] The fold effect indicated is based on the assays described in foonotes b) to k).
[b] Absence of HLA-DR, -DP, -DQ mRNA and protein in CIITA deficient cells.
[c] HLA-DO mRNA levels in Raji compared with RJ2.2.5.
[d] MHCI promoter activity (reporter gene assay) in transiently transfected HeLa cells.
[e] IL-4 production (ELISA) in CD4$^+$ T cells from CIITA knockout mice.
[f] FasL mRNA and surface expression in T cells from CIITA knockout mice, and in transfected Jurkat cells and T cell hybridoma.
[g] Collagen α2 (I) mRNA in IFN-γ-induced 2fTGH cells compared with G3A cells (defective in IFN-γ-induced CIITA expression).
[h] HIV expression (p24) and LTR promoter activity in Jurkat T cells stably transfected with CIITA and infected with HIV.
[i] HIV-LTR activity in 293T, HL3T1 and Jurkat cell lines cotransfected with CIITA and Tat.
[j] Plexin-A1 mRNA and protein in BM-DC from CIITA knockout mice.
[k] Microarray study comparing gene expression in Raji and RJ2.2.5 cell lines.

conditions [23, 24]. Finally, transfection experiments suggested that CIITA might suppress IL-4 expression in Th1 cells by competing with NF-AT, a key IL-4 gene transcription factor, for binding to the general coactivator CBP [25]. These results led to the model that CIITA could be a Th1-specific factor that functions as a repressor for IL-4 expression. However, our own studies do not support this model. We found that endogenous CIITA expression is not regulated differentially during Th1 and Th2 differentiation in human or mouse T cells [26]. Furthermore, ectopic expression of CIITA in T cells does not repress IL-4 expression in Th2 cells, but results instead in a strong Th2 bias during $CD4^+$ T cell activation [26]. The explanation for the discrepancy between our results and the earlier studies remains a matter of debate.

As with the IL-4 gene, FasL expression was shown to be increased in CIITA knockout mice [24]. Overexpression of CIITA in T cells was also found to lead to repression of the FasL gene [27]. As in the case of the IL-4 gene, the mechanism was proposed to involve a competition between CIITA and NF-AT for binding to CBP [27].

The collagen α2 (I) gene has been reported to be repressed by CIITA [28, 29]. One mechanism proposed for this repression again involves the sequestration of CBP by CIITA [28]. The same mechanism may also be implicated in down-regulation of the thymidine kinase and cyclin D1 genes [28].

The promoter in the long terminal repeat (LTR) of the HIV provirus has attracted attention as a novel target of transcriptional regulation by CIITA. Expression of CIITA was reported to increase LTR promoter activity and HIV replication in fibroblast and T cell lines [30, 31]. This was speculated to be relevant because CIITA is expressed in activated human T cells and macrophages, both of which are primary targets of HIV infection. However, although TGF-β is well known to inhibit the expression of CIITA, it instead stimulates HIV replication [32]. Furthermore, in other studies HIV infection and LTR activity were found to be reduced in $CIITA^+$ cell lines [33, 34]. Considering these conflicting findings it is presently not clear exactly how CIITA affects transcription driven by the HIV promoter *in vivo* under physiological conditions.

It has recently been reported that the semaphorin receptor plexin-A1 is expressed abundantly in mature mouse DC and that this expression is dependent on CIITA [35]. This observation is particularly interesting because plexin-A1 expression was found to enhance the ability of APC to promote T cell stimulation. The mechanism through which CIITA drives plexin-A1 expression remains obscure. The promoter of *Plxna1*, the gene coding for Plexin-A1, does not contain a characteristic W–X–X2–Y module [35], indicating that CIITA may be recruited by a mechanism distinct from that operating at MHCII genes. Alternatively, it has not been excluded that activation of the *Plxna1* promoter by CIITA could be indirect. The latter could explain why *Plxna1* expression is strongly increased after DC maturation [35] whereas the CIITA gene is in fact turned off during this process [36].

A recent microarray study has compared the gene expression profiles of the $CIITA^+$ B cell line Raji and its $CIITA^-$ counterpart RJ2.2.5 [37]. Over 40 genes whose expression appears to be modulated by CIITA were identified in this study. These genes have diverse functions, some of which could have an impact on antigen-processing, intracellular signaling or cell proliferation. However, compared with the MHCII genes, the changes in their expression levels between Raji and RJ2.2.5 were quite modest [37]. Moreover, none of these genes was found to contain the characteristic W–X–X2–Y module and physical recruitment of CIITA to the regulatory regions of these genes has not been demonstrated. It thus remains to be demonstrated that they are indeed *bona fide* targets of CIITA.

To determine whether the potential new target genes of CIITA are relevant *in vivo*, we have examined the expression of several of them in mice that express CIITA ubiquitously and in mice lacking CIITA in defined cell types (unpublished data). Mice expressing CIITA ubiquitously [26] do not display a significant change in expression of any of the putative novel target genes tested, whereas the MHCII and related genes are strongly up-regulated in different organs including the liver, lung and kidney. Furthermore, in contrast to the MHCII and related genes, expression of the proposed novel target genes was found to be independent of CIITA, as no reduction in their expression was observed in B cells or IFN-γ-induced fibroblasts from mice lacking CIITA in these cells (pIII+IV knockout mice; see below). The importance of CIITA for expression of the putative new target genes is thus not evident *in vivo*.

In conclusion, MHCII and related genes remain the major known targets of CIITA. There is growing evidence that CIITA may influence the expression of additional genes in certain cell types and under certain experimental conditions. However, it is not clear whether these genes are indeed *bona fide* targets of CIITA. In several cases CIITA may affect them via an indirect mechanism, for instance by sequestering another factor such as CBP. In other cases the molecular mechanisms involved remain unknown. Finally both the microarray analysis and our *in vivo* experiments in transgenic and knockout mouse models indicate that the contribution of CIITA to the expression of many of these genes is relatively minor at best.

3 Activation of CIITA expression

3.1 The *MHC2TA* gene is controlled by differential promoter activities

Expression of the gene encoding CIITA (*MHC2TA*) is controlled by four different promoters (pI to pIV) [38] (Fig. 2). Three of these promoters are highly conserved between the human and mouse genes (pI, pIII and pIV). However, pII has only been found in the human gene. It displays only very low transcriptional activity and its significance remains unknown. The different promoters do not share any sequence homology and are not co-regulated. They are distributed over a large (>12 kb) genomic region. Each promoter precedes a distinct first exon that is spliced alternatively to the shared downstream exons. This leads to the production of three types of transcripts (type I, type III and type IV) possessing different 5' ends [38] (Fig. 2).

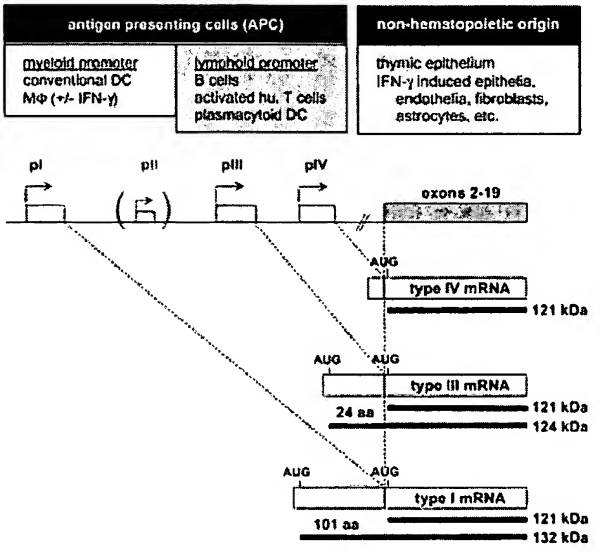

Fig. 2. Expression of the *MHC2TA* gene is controlled by three independent promoters having different functions: pI is active in cells of myeloid origin (conventional DC and IFN-γ-activated macrophages); pIII is used in cells of lymphoid origin (all B cell subsets, activated human T cells and pDC); pIV is essential for responsiveness to IFN-γ in non-BM-derived cells (endothelia, epithelia, fibroblasts and astrocytes) and is expressed constitutively in cTEC and mTEC. The three types of CIITA mRNA (white bars; types I, III and IV) initiated at pI, pIII and pIV encode three different protein isoforms (black bars; 121, 124 and 132 kDa). These proteins differ only at their N-terminal ends. Colored bars represent exons. The boundary between the alternative first exons and the shared downstream exons is indicated by a vertical line. The positions of translation initiation codons are indicated.

The shared second exon contains a translation initiation codon that can be used in all three types of transcript to give rise to a 1106 amino acid protein. However, the first exons of the type I and type III transcripts each contain an additional in-frame translation initiation codon. Usage of these alternative initiation codons leads to the synthesis of protein isoforms of 1207 and 1130 amino acids, respectively. The three CIITA isoforms have apparent molecular weights of 132 kDa, 124 kDa and 121 kDa (Fig. 2). All three protein variants exist *in vivo* [36, 39].

Cell-type-specific and modulated expression of the *MHC2TA* gene are controlled by the differential activities of the three promoters. It is thus the sophisticated transcriptional control of the *MHC2TA* gene that dictates the cell-type-specific and inducible expression of MHCII genes. The specificities of the *MHC2TA* promoters were initially determined by examining their usage and activity in cell lines and primary cells *in vitro*. More recently, two new mouse strains (pIV and pIII+IV knockout mice) have allowed us to define more precisely the function of each promoter *in vivo*. In pIV knockout mice, pIV is deleted but transcription from pI and pIII is unaffected [40]. In pIII+IV knockout mice, pIII and pIV have been excised and only pI-driven CIITA expression remains intact (unpublished data). In the following sections, we will discuss our current view of the differential usage of the CIITA promoters (Fig. 2).

3.2 CIITA usage among different cell populations

3.2.1 IFN-γ-stimulated cells

CIITA pIV is induced by IFN-γ in most cell types [38, 41–45]. A 300 bp promoter-proximal region is sufficient for the IFN-γ response [38, 41, 43]. This region contains a GAS element, an E box and an IRF-1-binding site, all three of which are required for induction in most cell types [38, 41, 42, 44, 46]. The first two are bound cooperatively by STAT-1 and upstream regulatory factor-1 (USF-1) [41, 42, 44]. STAT-1 is activated and translocated to the nucleus by the classical IFN-γ signal transduction pathway. USF-1 is a constitutively expressed member of the basic helix-loop-helix / leucine zipper family. The IRF-1-binding site of pIV is occupied by IRF-1. The synthesis of IRF-1 itself is induced by IFN-γ. This dependence on IRF-1 explains the delayed kinetics of CIITA induction by IFN-γ [47].

pIV knockout mice exhibit a highly selective abrogation of IFN-γ-induced MHCII expression on a wide variety of cells of non-hematopoietic origin, including endothelia, epithelia, astrocytes and fibroblasts [40]. This provides the formal proof that pIV is indispensable for

IFN-γ-inducible MHCII expression in non-professional APC.

In human fibrosarcoma and glioblastoma cell lines, a 5' flanking sequence situated approximately 5 kb upstream of the transcription initiation site of pIII has been reported to confer IFN-γ responsiveness [42]. The functional relevance of this sequence is supported by the presence of a DNaseI hypersensitive site at this position *in vivo* [43]. The putative regulatory region acts as a STAT-1-dependent and IRF-1-independent enhancer [43]. In contrast to these findings in human cells, pIII does not seem to be inducible by IFN-γ in primary rat astrocytes [48] or in mouse macrophages [40, 49]. Moreover, pIII is not sufficient for driving IFN-γ-induced CIITA and MHCII expression in non-BM-derived cells of the pIV knockout mice [40]. There may thus be a species-specific difference in the IFN-γ-responsiveness of pIII.

3.2.2 Macrophages

The first analysis of CIITA promoter usage in macrophages was based on the examination of monocyte/macrophage cell lines. IFN-γ-induced human THP-1 cells were for instance found to express CIITA type IV transcripts [38]. However, it is now clear that pIV is not the most important inducible promoter in macrophages. IFN-γ-induced macrophages of both the pIV and pIII+IV knockout mice express MHCII molecules at normal levels, indicating that pIV is in fact not essential in these cells ([40] and unpublished data).

Instead, the key promoter for sustaining CIITA and MHCII expression in IFN-γ-induced macrophages is pI rather than pIV. This is in sharp contrast to non-BM-derived cells, which remain MHCII$^-$ in the pIV and pIII+IV knockout mice (see above), indicating that they are strictly dependent on pIV. In wild-type macrophages, both CIITA type I and type IV transcripts are induced by IFN-γ at early time points [49]. However, at later time points, type IV mRNA declines and CIITA expression is dominated by type I mRNA, which remains elevated for long periods of time [40, 49]. Induction of pIV is thus only transient while that of pI is sustained. It remains unknown how IFN-γ activates pI. No IFN-γ-responsive sequences have been identified in the vicinity of pI. It is thus possible that IFN-γ affects pI indirectly as a consequence of macrophage activation. The answer to this question awaits further dissection of the regulatory mechanisms controlling pI.

3.2.3 TEC

MHCII molecules are constitutively expressed on cTEC and drive the positive selection of CD4$^+$ T cells [1]. Unexpectedly, cTEC of pIV knockout mice are MHCII$^-$ [40, 50]. This loss of MHCII expression results in the abrogation of positive selection of CD4$^+$ T cells in the thymus [40, 50]. CD4$^+$ T cell counts in the thymus and in the periphery of pIV knockout mice are reduced as strongly as in MHCII knockout mice. Thus pIV is absolutely essential for constitutive expression of CIITA in cTEC. It is also required in mTEC [50]. The molecular mechanism mediating pIV activation in TEC is not known. It must be independent of the IFN-γ signaling pathway, because knockout mice lacking key components of this pathway, such as the IFN-γ receptor, STAT-1 and IRF-1, have normal MHCII expression on cTEC and unperturbed positive selection of CD4$^+$ T cells [50].

3.2.4 B cells

Transcription of the *MHC2TA* gene in B cells is initiated from pIII. This was already evident from early studies using B cell lines [38, 51]. Our recent analysis of the pIII+IV knockout mice has provided the final proof that pIII is indeed essential for expression of the *MHC2TA* gene in all B cells *in vivo*, including B-1 and B-2 cells in the spleen, thymus, blood and peritoneum (unpublished data). A 320 bp promoter-proximal regulatory region of pIII is sufficient for the B-cell-specific activity of pIII [38, 51]. This region contains five sequence elements that have been shown by genomic footprinting experiments to be occupied *in vivo* in B cells [52]. At least two of these elements — activation response element (ARE)-1 and ARE-2 — are critical for proper activity [52]. In addition, the 5' untranslated region of pIII seems to function as an important regulatory region in B cells [53].

3.2.5 T cells

Activated human T cells express MHCII molecules [54, 55]. This expression is regulated by CIITA induced from pIII [55, 56]. Mouse T cells have also been shown to express low levels of CIITA after activation [23, 24, 26], but this expression is not sufficient to induce the presence of MHCII molecules at the cell surface [26, 57]. As discussed before, in the section on the specificity of CIITA, there is a controversy concerning the differential expression of CIITA in Th1 and Th2 cells. Although others have proposed that CIITA is expressed in Th1 but not Th2 cells [23, 24], we have found no difference in CIITA expression between Th1 and Th2 cells either in humans or mice [26]. Certain differences have been observed between the molecular regulation of pIII in B cells and

human T cells [55, 56]. These reflect a difference between the pathways leading to constitutive expression in B cells and induced expression in activated T cells.

3.2.6 Melanoma cells

Some melanoma cells display an unusual constitutive expression of MHCII molecules. This aberrant pattern of expression is due to the constitutive activation of *MHC2TA* pIII [58, 59]. It has been proposed that the 5' flanking sequence conferring IFN-γ responsiveness to pIII in human fibrosarcoma and glioblastoma cell lines [42] is implicated in constitutive expression of pIII in these melanoma cells [58].

3.2.7 DC

In DC, pI is the promoter used predominantly for CIITA expression. CIITA type I transcripts always represent a preponderant fraction in various DC preparations including *ex vivo* mouse splenic and thymic DC, mouse BM-derived DC, long-term mouse DC cultures and human monocyte-derived DC ([36, 38, 40, 60] and unpublished data). However, CIITA type III transcripts are also found in significant amounts in human monocyte-derived DC [36]. Surprisingly, the recently discovered plasmacytoid DC (pDC) or interferon-producing cells [61] are completely devoid of CIITA, and thus MHCII expression, in mice lacking pIII (unpublished data). In agreement with this observation we have found that the expression of CIITA in pDC, unlike all other conventional DC subsets, is controlled by pIII rather than pI. Thus, pDC can be uncoupled from conventional DC in terms of their molecular regulation of CIITA and MHCII expression. This is consistent with the notion that pDC may be more closely related to the lymphoid cell lineage [62].

In summary, the pIV and pIII+IV knockout mice have provided definitive evidence that differential CIITA promoter usage does indeed play an important physiological role (Fig. 2). Cells of myeloid origin (conventional DC and macrophages) rely mainly on pI for constitutive or IFN-γ-induced CIITA expression. In cells of lymphoid origin (B cells, T cells and pDC) CIITA expression is driven almost exclusively by pIII. Finally, pIV is indispensable for IFN-γ-activated expression in non-professional APC and for expression in TEC. The three *MHC2TA* promoters are independent of each other and there appears to be no cross-talk between them. This point is demonstrated by the fact that individual promoters can be excised from the genome without affecting the specificity or activity of those that are retained.

4 Silencing of CIITA expression

4.1 Repression of the *MHC2A* gene is important in physiological and pathological situations

It has become increasingly evident over the past few years that repression of the CIITA gene plays a key role in the down-regulation of MHCII expression in various physiological and pathological situations. First, the down-regulation of MHCII expression is observed in normal cells and tissues and this is likely to be important for homeostasis of the immune system, for the regulation of immune responses and for avoiding autoimmunity. Second, there is growing evidence that tumor cells may escape recognition and elimination by the host immune response by silencing MHCII expression. Finally, several pathogens have acquired mechanisms to evade immune surveillance by inhibiting MHCII expression. As a general rule, the inhibition of MHCII expression in these situations is mediated by repression of the *MHC2TA* gene (Fig. 1).

4.2 Down-regulation of MHCII expression in normal cells

4.2.1 CIITA silencing during DC maturation

In response to a variety of stimuli, such as infections by bacteria or viruses, immature DC are induced to undergo profound changes in their morphology and function. Changes in the synthesis, peptide-loading and cellular localization of MHCII molecules represent key aspects of this maturation process. The density of MHCII molecules expressed at the cell surface is increased as a result of changes in the intracellular localization and stability of pre-existing MHCII proteins. In contrast, *de novo* synthesis of MHCII molecules is shut down. This reduction in MHCII synthesis during DC maturation is a consequence of a rapid transcriptional inactivation of the *MHC2TA* gene [36]. This is mediated by a global repression mechanism implicating histone deacetylation over a large domain spanning the entire *MHC2TA* regulatory region [36].

4.2.2 CIITA silencing in plasma cells

Expression of the *MHC2TA* gene is actively silenced during terminal differentiation of B cells into plasma cells [63, 64]. The sequence elements of pIII that are occupied in normal B cells are completely bare in plasma cells [52, 63]. The human positive regulatory domain I binding factor-1 (PRDI-BF1) [65] and its mouse homologue B-lymphocyte-induced maturation protein-1 (Blimp-1) [66]

have been proposed to play a crucial role in the repression of pIII in plasma cells [67, 68]. PRDI-BF1/Blimp-1 is up-regulated when B cells differentiate into plasma cells [69]. It has also been shown to drive, at least partially, the final differentiation of B cells into plasma cells if expressed ectopically in BCL1 lymphoma cells [69]. PRDI-BF1/Blimp-1 can bind *in vitro* to a sequence situated in the promoter-proximal region of pIII [67, 68]. However, no occupation of this site is seen in *in vivo* footprint experiments performed with plasma cells [52]. The mechanism by which PRDI-BF1/Blimp-1 silences the *MHC2TA* gene thus remains to be clarified.

4.2.3 Inhibition of IFN-γ-induced CIITA expression

IFN-γ-activated expression of CIITA can be suppressed by a number of different stimuli including TGF-β, IL-1, IL-4 and IL-10 [45, 70–72]. TGF-β markedly attenuates IFN-γ-induced CIITA expression. The inhibitory mechanism involves inhibition of *MHC2TA* transcription [70–72]. Surprisingly, TGF-β does not affect IFN-γ-induced phosphorylation of JAK-1, JAK-2 or STAT-1. Nor does it interfere with binding of STAT-1, USF-1 or IRF-1 to pIV of the *MHC2TA* gene [73, 74]. Moreover, TGF-β even inhibits basal non-induced expression levels of pIV [42, 75]. Finally, the activity of the putative IFN-γ response element situated upstream of pIII is also inhibited by TGF-β [43]. Dong et al. have reported that Smad-3 is essential for the inhibitory effect of TGF-β on CIITA expression [74]. IL-1, IL-4 and IL-10 have also been shown to exert an inhibitory effect on CIITA transcription in human astrocytes (IL-1) [45] and in mouse microglia (IL-4 and IL-10) [72]. The role of IL-4-mediated CIITA inhibition seems to be cell-type-dependent.

IFN-γ-induced gene activation is generally a transient event. Suppressor of cytokine signaling-1 (SOCS-1) has been shown to be induced by IFN-γ and this protein negatively regulates the IFN-γ signal transduction pathway by binding to JAK-2 and inhibiting its kinase activity [76, 77]. SOCS-1 can thus also suppress IFN-γ-activated expression of pIV of the *MHC2TA* gene [46]. Similar to SOCS-1, nitric oxide — which is produced by macrophages upon IFN-γ stimulation — may act as a feedback inhibitor of MHCII synthesis by inhibiting INF-γ-induced CIITA expression [78].

Statins (HMG-CoA reductase inhibitors), which are well known for their cholesterol-lowering effect, have been shown to exhibit a number of anti-inflammatory properties. Among other effects, they repress IFN-γ-induced MHCII expression by inhibiting activation of the *MHC2TA* gene [79, 80]. Statins may thus be of potential interest as a treatment in clinical situations where repression of MHCII-dependent T cell activation is desired. Such situations include immunosuppression following organ transplantation or autoimmune diseases such as multiple sclerosis and rheumatoid arthritis [79–81].

4.2.4 CIITA silencing in trophoblasts

Fetal trophoblasts lack expression of MHCII molecules, both constitutively and after exposure to IFN-γ. The absence of MHCII molecules on trophoblasts is thought to play a critical role in preventing rejection of the fetus by the maternal immune system. The inability of trophoblasts to express MHCII genes is primarily due to the lack of CIITA expression [82, 83]. It has been shown that pIV is hypermethylated at CpG dinucleotides in trophoblast cell lines and primary trophoblasts [84, 85]. This has been shown to block the activation of pIV by inhibiting the binding of STAT-1 and IRF-1 as well as the ensuing chromatin remodeling [86]. Recently, an additional intriguing mechanism has been proposed to be implicated in MHCII silencing in trophoblasts [87]. It involves a trophoblast-derived non-coding RNA that is able to suppress IFN-γ-induced CIITA expression through an inhibitory domain in pIV.

4.3 CIITA silencing in tumor cells

MHC molecules play a pivotal role in presenting tumor-derived antigens and hence in activating and regulating antitumor immune responses [88, 89]. Consequently, one strategy employed by malignant cells for evading recognition and elimination by the immune system involves the loss or down-regulation of MHC expression [88, 89]. The partial or complete loss of MHCI expression is frequently observed because cytotoxic $CD8^+$ T cells, which recognize antigenic peptides presented by MHCI molecules, constitute the primary effector cells mediating tumor rejection [90]. However, efficient and long-lasting antitumor immunity requires help provided by $CD4^+$ cells during both the priming and effector phases of the antitumor response [91, 92]. The loss of MHCII expression on malignant cells is thus also a commonly observed escape strategy.

The loss of constitutive MHCII expression is observed in tumor cells of hematopoietic origin, particularly in B and T cell malignancies [93–95]. Moreover, the inability to induce MHCII expression in response to IFN-γ is often associated with tumor cells of non-hematopoietic origin [58, 95–100]. There is growing evidence that this inability to express MHCII results from epigenetic silencing of the *MHC2TA* gene [95–102]. The regulatory regions of the

MHC2TA gene have been found to be hypermethylated at CpG dinucleotides in MHCII$^-$ T cell leukemias, B cell lymphomas and various tumor cells that are unable to express MHCII upon exposure to IFN-γ, including teratocarcinoma, choriocarcinoma, neuroblastoma, erythroleukemia and small cell lung cancer [95, 97, 100–102]. Histone deacetylation rather than DNA hypermethylation has been implicated in silencing of *MHC2TA* expression in several squamous cell carcinoma cell lines [103].

4.4 Repression of CIITA expression by pathogens

Pathogens have developed a wide variety of strategies to escape immune surveillance by their hosts [104, 105]. In order to inhibit the establishment of a protective immune response, several bacteria and viruses down-regulate MHCII expression and thus prevent the activation of specific CD4$^+$ T cells. They achieve this by interfering with the function or expression of CIITA.

The intracellular bacterium *Chlamydia* down-regulates CIITA expression by inducing the degradation of USF-1, a ubiquitous factor that is required for the activation of pIV of the *MHC2TA* gene [106]. Infections with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), *Mycobacterium tuberculosis* (MTB) or *Toxoplasma gondii* also down-regulate CIITA expression, but the precise mechanisms that are involved remain poorly defined [107–109]. The MTB 19-kDa lipoprotein inhibits IFN-γ-induced MHCII expression in macrophages by preventing the induction of CIITA type I and type IV mRNA. Interestingly, it seems to exert its negative effect on IFN-γ signaling by inhibiting IRF-1, independently of SOCS-1 and STAT-1 [108].

Varizella zoster virus, human cytomegalovirus (CMV) and human parainfluenza virus type 3 (HPIV3) also inhibit IFN-γ-induced CIITA expression [110–113]. Varizella zoster virus does so by interfering with the IFN-γ signaling pathway. It blocks STAT-1α and JAK2 expression and therefore transcription of the downstream IRF-1 and CIITA genes [110]. Infection with CMV can direct JAK-1 to the proteasome for degradation or affect the IFN-γ signaling pathway downstream of STAT phosphorylation and nuclear translocation [111, 112]. The inhibitory effect of HPIV3 on IFN-γ-induced CIITA expression is due to a defect downstream of STAT-1 activation, but the precise mechanism remains unclear [113]. Finally, as mentioned earlier, the HIV Tat protein can inhibit MHCII expression by interfering with the function of CIITA in HIV-infected or Tat-transfected fibroblasts and T cell lines [34, 114]. Tat does this by competing with CIITA for binding to cyclin T1, a component of the transcriptional elongation complex P-TEFb [34, 114].

Taken together, these findings demonstrate that many pathogens have acquired a means to target the IFN-γ signal transduction pathway. Among other consequences, this results in the inhibition of IFN-γ-induced CIITA and MHCII expression, which may favor the escape from immune surveillance and thus facilitate the establishment of persistence. It will be of great interest to dissect in greater detail the strategies used by pathogens to interfere with CIITA and MHCII expression. This may contribute to the design of new approaches for fighting these pathogens.

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Genetic Control of MHC Class II Expression

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The presentation of peptides to T cells by MHC class II molecules is of critical importance in specific recognition by the immune system. Expression of class II molecules is exquisitely controlled at the transcriptional level. A large set of proteins interact with the promoters of class II genes. The most important of these is CIITA, a master controller that orchestrates expression but does not bind directly to the promoter. The transcriptosome complex formed at class II promoters is a model for induction of gene expression.

One of the keys to the development of a specific immune response to a pathogen is held by MHC class II molecules (Cresswell, 1994; Nelson and Fremont, 1999). Unlike class I membrane glycoproteins, which are widely expressed, class II molecules are generally restricted to a subset of antigen presenting cells, such as macrophages, dendritic cells, and B cells. Their expression can be induced on other cells types after stimulation with cytokines such as interferon γ. MHC class II molecules are responsible for presenting peptides derived from extracellular pathogens to T cells bearing the CD4 marker. There are three classical class II molecules in man: HLA-DP, -DQ, and -DR. Mice only express proteins orthologous to the last two, A and E, respectively. In addition to these structures, both species encode so-called nonclassical molecules, namely HLA-DM and -DO in man, and M and O in mouse. These molecules do not normally reside at the cell surface, and they do not present antigens; instead, they modulate binding of peptides to the classical structures. Each class II molecule is a heterodimer of an α chain and a β chain. The transcriptional control of this family of genes has been extensively studied. Numerous DNA binding transcription factors as well as a master coactivator (CIITA, class II transactivator) have been identified. A clear picture of the roles of these factors in the induction of chromatin changes and in the formation of an active transcriptosome has emerged, rendering this a model system to study these issues. In addition, mounting evidence shows that the regulation of class II MHC genes is highly relevant to some important diseases. This review provides a brief background to the genetics, structure, and function of class II, and then focuses on regulation of expression of MHC class II genes.

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Class II Region and Genes

The α and β chains of each class II molecule are encoded by separate genes in the class II region of the MHC (Figure 1A) (Allcock et al., 2000; Beck and Trowsdale, 1999; Gunther and Walter, 2001). In all cases, except for *HLA-DO*, the pairs of genes are encoded adjacently. Some of the genes are duplicated, one copy of each being functional in the case of *DP* and *DQ*. *DRB* is a special case, as there can be more than one functional copy per haplotype, in addition to nonfunctional pseudogenes. Each mouse haplotype also contains two H-2M β chain genes, *Mb1* and *Mb2*, both of which are functional. Class II sequences obviously arose by repeated duplications. These must have taken place at several different periods throughout evolution of the class II gene family. The DM sequence is only weakly related to other class II sequences and probably resulted from an ancient gene duplication. In contrast, DO sequences are ~60% identical to DR. *DRB* loci are highly similar and must represent recent duplications.

The class II region of the human and rodent MHCs also harbors a small group of genes involved in antigen processing, which encode the TAP transporters as well as interferon-induced proteasome components. Some class I genes are tightly linked to the class II region, at the centromeric end of the MHC, in rodents, but not in humans. This end of the extended class II region also contains the gene for TAPASIN, which is involved in antigen processing for loading class I molecules.

A feature of the MHC is the high degree of linkage disequilibrium across the complex, and the region is divided into extended units, or haplotypes (Dawkins et al., 1999). It is not established whether this is maintained by selection, polarized recombination, or founder effects, but genetic recombination in the MHC class II region is highly focused into hotspots (Cullen et al., 1997; Jeffreys et al., 2001). In terms of class II, explanations could be invoked for maintaining certain combinations of alleles of different genes in *cis* relationship. Both chains of HLA-DQ and H-2A are polymorphic, and particular DQ and H-2A α and β chains do not pair efficiently. In most populations studied to date, one rarely finds α and β alleles encoding these unstable heterodimers on the same haplotype. For example, DQw1-associated β chain is not found together with a DQw-2, -3, or -4 associated α chain and vice versa. The haplotypes containing these unstable heterodimers are generally thought of as "forbidden." Some exceptions to this general rule may be identified, especially in small isolated populations (Grahovac et al., 1998).

Class II Proteins

Each class II heterodimer consists of two integral membrane proteins of around 26 kDa (Figure 1B). The differences in size of the α (32 kDa) and β (29 kDa) chains are mainly attributed to differences in N-linked glycosyl-

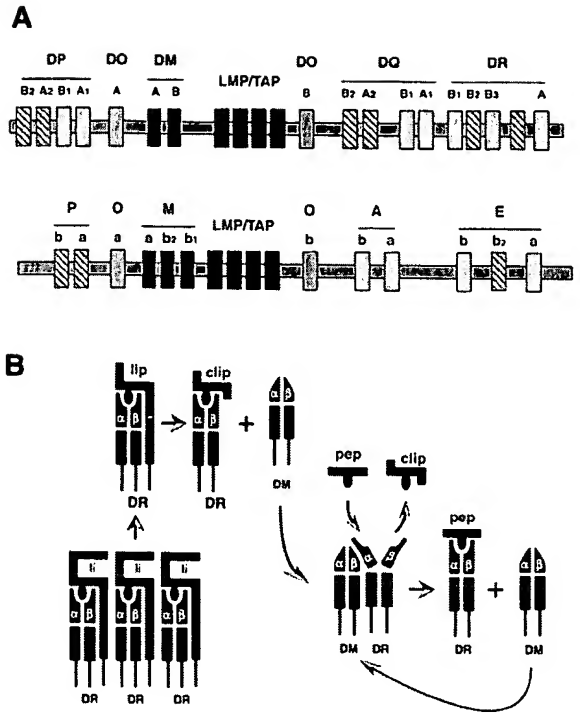

Figure 1. Genetics and Loading of MHC Class II

(A) Schematic maps of the MHC class II regions in man and mouse. The main genes are shown, including classical class II molecules (yellow). Pseudogenes are hatched. Nonclassical class II genes are pink (*DO*) and dark blue (*DM*). Antigen-processing genes for loading peptides onto class I molecules are in purple and green.

(B) Simplified mechanism for DM-mediated peptide exchange on DR molecules. Class II dimers assemble with Ii in the ER to form a nonameric complex of an Ii trimer and three class II dimers. The complexes are transported to specialized lysosome-like compartments for loading of antigenic peptides. In these vesicles, the Ii chain is hydrolyzed to leave class II bound to the Ii derivative, CLIP. DO (not shown) is also associated with DM in the ER and it travels with DM. Peptide exchange is catalyzed by DM, by stabilizing the transition state.

ation. The α and β chains of all classical class II molecules have the same overall conformation, each consisting of two extracellular domains, α1 and α2, and β1 and β2, respectively. The membrane-distal domains combine to form a single peptide binding site composed of two antiparallel α-helical loops supported by a platform of eight antiparallel β strands. These domains feature the high level of polymorphism exhibited by MHC molecules. A conserved disulfide bond connects the α-helical region of the β1 domain to a strand in the platform floor (β10cys to β78cys). The DM molecule contains two additional disulfide bonds (α 24-79 and β 25-35).

The groove of class II, like that of class I molecules, is capable of binding a wide range of peptides. Peptides bind to class II in an extended conformation. In contrast to class I, the N and C termini of class II-bound peptides may extend beyond the ends of the groove. The peptide is held by a series of hydrogen bonds between the peptide backbone and conserved amino acid side chains lining the groove. Since the bonds do not involve peptide side chains, they confer sequence-independent binding. This may explain how class II molecules can bind multiple peptides with high affinity and low specificity. The three-dimensional structure of the nonclassical class II molecule, HLA-DM, reveals its unique function (Alfonso and Karlsson, 2000; Mosyak et al., 1998). The peptide binding site is altered to an almost fully closed groove, and the α helixes of the α1 and β1 domains contact each other over the first and last thirds of their length. A cavity remains at the center of the membrane-distal portion of the molecule, forming a deep, polar pocket, 10 Å wide and 10 Å deep. This pocket is conserved in orthologous molecules from other species, such as H-2M. It could bind the end of a peptide, but is not as large as the lipid binding CD1 pocket. The molecule also has a tryptophan-rich lateral surface that may bind the other nonclassical class II molecule, HLA-DO. MHC class II molecules pack as pairs of heterodimers in some crystals, and the possibility of "dimers of dimers" forming at the cell surface has fuelled speculation about the stoichiometry of interaction with T cell receptors. Evidence for dimer pairs is controversial (Schafer et al., 1995).

Polymorphism

Classical class II sequences exhibit an extraordinary degree of variation that is concentrated on the amino acid residues that shape the peptide binding site. The involvement of selection in the maintenance of the polymorphism is suggested by the finding of a high level of nonsynonymous codon changes. This is in contrast to most other genes, as well as the membrane-proximal domains (α2 and β2), where synonymous variation normally predominates. Except for *DQA* and *Aa*, the sequences encoding the α chains are generally less variable. There are few alleles of *Ea* and *DRA*, with conservative amino acid changes. Generation of the polymorphism could be due to point mutation, but the mutation rate is not especially inflated compared to more conventional genes. It is likely that "allele conversion" (double crossover to replace a short section of an allele) has taken place repeatedly, because the alleles have the semblance of being "patchworks" of each other. Some alleles could have arisen by recombination, using single crossovers. Gene conversion has also been proposed as a possible mechanism for incorporating sequences from other linked class II genes. There is evidence for this mechanism in conversion of class I sequences in mice.

The nonclassical class II molecules are relatively invariant. Some alleles of both *HLA-DM* and *-DO* have been described, but these vary by small numbers of amino acids and, so far, have no known functional significance.

Peptide Loading, Ii, and the Role of DM

Soon after synthesis, classical class II molecules associate in the endoplasmic reticulum (ER) with a third protein, a type II (i.e., of inverted orientation) membrane glycoprotein called invariant chain (Ii) (Cresswell, 1996) (Figure 1B). The combined proteins form a nonameric structure, consisting of three Ii chains, arranged as a core, surrounded by three classical class II heterodi-

mers. The grooves of the class II molecules are occupied, in the nonamer, by a section of the Ii chain called CLIP. This may help to avoid loading of the groove with ER-resident proteins, as is the case for class I. The main function of invariant chain seems to be as a chaperone to ensure correct folding and egress of class II. It contains a di-leucine targeting signal in its cytoplasmic tail which helps to divert the nonamer from the default secretory pathway to lysosomal-like vesicles, called MIIC (for MHC class II compartment), where peptide is eventually loaded. Before this can take place, however, Ii is degraded by lysosomal proteases such as cathepsin L and S. It is progressively cleaved, leaving just the CLIP fragment itself occupying the groove.

The MIICs are depots where the class II molecules, groomed in this way, meet up with antigenic peptides that come from degradation of exogenous proteins. These may be internalized either by endocytosis or by interaction with surface receptors on antigen presenting cells (APCs). For example, proteins bound to surface antibodies are internalized on B cells. Other cells may take up antibody:antigen complexes using the range of Fc receptors. Lectin-like receptors, such as mannose receptors, may be invoked to deal with glycoproteins. Topologically, peptide loading in MIIC vesicles is "outside" in that it is separated by membrane from the cytoplasm.

Efficient exchange of CLIP for antigenic peptides is mediated by DM (Sanderson and Trowsdale, 1995). The structure of the DM molecule reveals that it is highly unlikely to bind peptides and the groove of DM is effectively sealed (Mosyak et al., 1998). Moreover, at steady state, most of the DM molecules reside in the MIIC vesicles. DM binds transiently to class II:CLIP and stabilizes an intermediate state where CLIP is released, allowing other peptides to bind. A speculative model proposes that DM contacts DR "shoulder to shoulder": a conserved tryptophan residue (α62 Trp) in DM interacts with α51 Phe of DR, at the extended strand where the class II groove differs from that of class I, near pocket 1 (Nelson and Fremont, 1999; Doebele et al., 2000). This interaction could result in destabilization of several peptide:MHC bonds, lowering the free energy barrier to peptide dissociation. DM stabilizes the open transition conformation of DR, favoring faster peptide association, in the MIIC environment that is rich in imported, antigenic peptides. The class II molecule may be quite flexible around the first, hydrophobic pocket in the absence of bound antigenic peptide. A more rigid conformation is probably generated after filling of pocket 1, which would render the molecule less susceptible to the effects of DM (Chou and Sadegh-Nasseri, 2000). The side chains in CLIP could be structured in such a way as to permit binding to all classical class II molecules but release from the groove under appropriate conditions, such as in the presence of DM and in the low pH of the MIIC vesicle. CLIP can be regarded therefore as a disposable stuffer. Once antigenic peptide is stably bound, DM may lose its association for class II. Alternatively, DM may be released at the cell surface, to be retargeted to MIICs. The cytoplasmic tail of DMβ contains a tyrosine-based targeting signal (Copier et al., 1996).

The complex of classical and nonclassical class II molecules in the MIICs associates with tetraspan molecules CD63 and CD82, which may also play a role in the later stages of class II maturation (Hammond et al., 1998).

The Role of DO

The second nonclassical class II molecule HLA-DO arose at a later stage of evolution to HLA-DM (Haas et al., 1987). DO is also monomorphic and, like DM, it resides in MIICs. It appears to require association with DM to access these vesicles. In some hands, DO appears to counteract the effect of DM, in a pH-dependent manner. Its effects may be optimal at pH 6, blocking peptide exchange in early endosomes. DO does not work as well at pH 5, the condition which favors DM-mediated peptide exchange in MIIC vesicles. A simple model to account for the action of DO would be for both DR and DO to compete for the same site on DM.

There is no consensus on the precise function of DO, and in some experiments it seems to enhance peptide exchange (Kropshofer et al., 1999). A clue to the function of DO may be provided by its expression, which is restricted mainly to B cells. Indeed, control of transcription of *DOB* may be less dependent on the CIITA transcription factor and induction by IFN-γ (see below). In B cells, DO may help to refine peptide loading to a restricted subset of class II molecules.

Expression of Class II

Class II molecules are constitutively expressed on cells that serve as APCs for CD4+ T cells, such as macrophages, monocytes, dendritic cells, and B cells; they may be induced on other cells by IFN-γ. Class II expression is also modulated by other agents, such as IL-4, IL-10, IFN-α/β, TNFα, and glucocorticoids. Concomitant expression of all three classical molecules is usually observed, although exceptions exist. Some B cells express solely DQ and others only DR. This raises the important question of whether the different class II isotypes exert distinct T cell functions, or whether they merely enlarge the peptide binding repertoire. Distinct functions have been suggested for HLA-DQ and -DR, the former being more likely to "suppress" some responses (Hirayama et al., 1987). These observations may enjoy renewed interest now that suppression of T cell responses by T cells has finally gained credibility. This issue is of crucial importance in view of the association of class II loci with a vast array of autoimmune conditions—an association which is still not fully explained.

Clearly, precise regulation of class II expression is critical. To address this, much effort has been devoted to the analysis of MHC class II promoters and the transcription factors that are involved in their regulation. The following sections are devoted to these aspects of MHC class II.

Promoter Motifs

One of the outstanding features of MHC class II loci is that not only the structural genes, but also the promoter elements, are remarkably conserved. All the classical and nonclassical class II promoters, including that for Ii, contain three elements—S (also W or Z), X, and Y—which are necessary for optimal constitutive and cytokine-induced gene expression. These sequences have

Table 1. Representative MHC Class II Defective Cell Lines

Complementation group	Patient-derived cell lines	In vitro mutants	MHCII Ag	MHCII promoter activity/mRNA	Genetic defect	RFX binding	Promoter occupancy
A	BLS-2, BCH1,2	RJ2.2.5	–	–	*MHC2TA*	+	+
B	BLS-1, Ra	–	–	–	*RFXANK*	–	–
C	SJO	G1B (IFN-γ)	–	–	*RFX5*	–	–
D	DA, ABI	6.1.6	–	–	*RFXAP*	–	–
Atypical lines							
		G3A (IFN-γ)	–	–	unknown	+	–
	KEN/KER		– for DRB, DQB, DPA	–	unknown	+	– for *DRB, DQB, DPA*

been extensively reviewed elsewhere (Benoist and Mathis, 1990; Glimcher and Kara, 1992), and will not be discussed here in detail. In addition to sequence conservation, the stereospecific alignment (i.e., DNA helical orientation and spacing) of the three elements is also critical (Harton and Ting, 2000). These data strongly implicate a model in which proteins binding to the S/W, X, and Y elements must bind in a spatially-restricted fashion to allow direct interactions among them, and/or interaction with a coactivator to form the active transcriptosome complex (see below).

Bare Lymphocyte Syndrome (BLS)

A discussion of the field of MHC class II regulation would not be complete without considering the heterogeneous group of genetic disorders, collectively called BLS or MHC class II deficiency (MIM209920). Several excellent reviews have appeared elsewhere (DeSandro et al., 1999; Reith and Mach, 2001), and only a brief discussion directly relevant to this review follows. BLS is a rare immunodeficiency inherited as an autosomal recessive disease; it arises due to a high degree of consanguinity in patients' families. Patients typically suffer from frequent, severe bacterial, fungal, or viral infections. Cells from the typical BLS patient lack constitutive and inducible expression of all MHC class II genes, including the α and β chains of DR, DP, and DQ. These patients exhibit severely hampered T cell activation and greatly reduced $CD4^+$ cells, although a recent report has described a family with a L469P mutation in CIITA that presents as an attenuated clinical course accompanied by residual MHC class II expression (Wiszniewski et al., 2001). In all cases that were tested, the MHC class II genes were not structurally defective, since fusions between defective cells and a normal cell invariably lead to surface expression of class II from the genotypes of both cells. EBV-transformed B cell lines obtained from these patients have proven invaluable in deciphering the regulatory pathway of MHC class II genes (Table 1; partly adapted from Reith and Mach, 2001, with permission, from the Annual Review of Immunology, Volume 19. ©2001 by Annual Reviews, www.annualreviews.org). In addition to B cell lines obtained from BLS patients, several mutant cell lines have been generated in vitro, primarily based on the lack either of constitutive MHC class II expression or of IFN-γ-induced MHC class II expression. Somatic cell fusions of BLS-derived cell lines and/or in vitro generated class II-negative cell lines have led to the delineation of four complementation groups. Additionally, two atypical cases represented by twins (KEN/KER) in whose B cells *DRB, DQB,* and *DPA* are not expressed have been reported (Douhan et al., 1996; Hauber et al., 1995). The in vitro generated MHC class $II^{-/-}$ cell line, G3A, also represents an atypical case. In this cell line, although X and Y binding proteins appear to be normal, CIITA induction by IFN-γ is not optimal, and the introduction of exogenous *MHC2TA* restores class II expression (Chin et al., 1994). Except for these atypical cases, where the genetic defect is undefined, it is now clear that each complementation group has a specific defect in a transcription factor that is necessary for MHC class II expression (see below) (Reith and Mach, 2001).

Transcription Factors and Coactivator

The initial characterizations of proteins that bind directly to MHC class II promoters identified both constitutively and ubiquitously expressed factors (Figure 2A). The Y element, a canonical CCAAT box, is bound by NF-Y/CBF, a molecule that is conserved from yeast to human (Maity and de Crombrugghe, 1998; Mantovani, 1999). NF-Y binds to DNA as a heterotrimer consisting of A, B, and C subunits. The B and C subunits contain histone-fold motifs that are similar to eukaryotic histones H2A and H2B and an archaebacterial histone-like protein. The RFX factor, also a trimer, binds to the X1 element (Durand et al., 1997; Masternak et al., 1998; Nagarajan et al., 1999; Steimle et al., 1995). It consists of RFXANK/RFXB, RFX5, and RFXAP, and defects in each define the BLS complementation groups B, C, and D, respectively (Table 1). RFX5 belongs to the RFX family of DNA binding proteins, and it was identified by complementation cloning using the MHC class II defective cell line, SJO (Steimle et al., 1995). RFX5 has a DNA binding domain and a C-terminal domain that interacts with NF-Y (Reith and Mach, 2001). The other two components of the complex that bind X1 were identified by biochemical purification. RFXANK/RFXB has ankyrin repeats typically thought of as mediating protein-protein interactions. These repeats provide an interaction platform to assemble the RFX complex by interacting with RFXAP, RFX5, and CIITA. A single nucleotide mutation in the ankyrin repeats results in abrogation of the RFXANK-RFXAP interaction in a BLS cell line, affirming the importance of these repeats (Nekrep et al., 2001). RFXAP, or RFX-associated protein, contains acidic, basic, and glutamine-rich sequences (Masternak et al., 1998). A recent report has shown that only the C terminus of RFXAP is essential for function, and different segments within this region are required for allele-specific class II expression

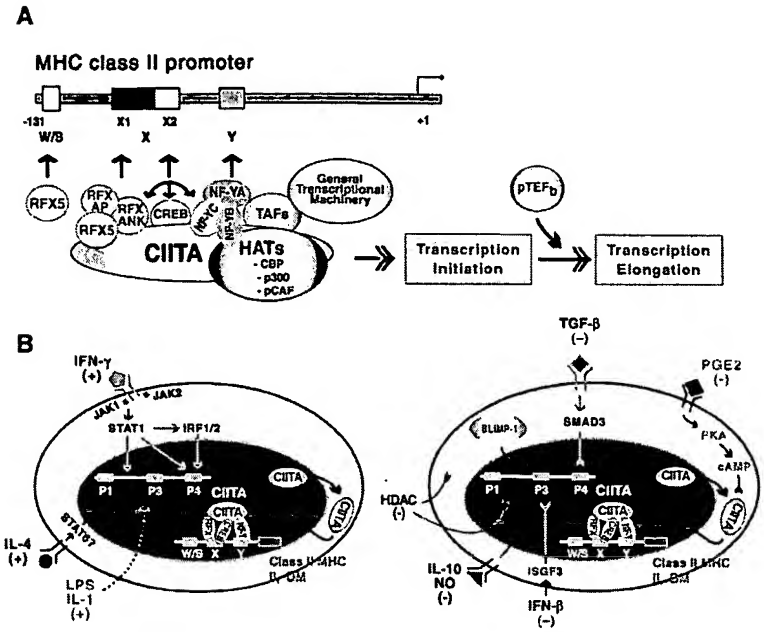

Figure 2. Molecular Regulation of MHC Class II

(A) A prototype MHC class II promoter and transcriptosome. The MHC class II promoter, its cognate DNA binding factors (NF-Y, CREB, and RFX), and the coactivator, CIITA, are shown. Positioning of the RFX subunits is drawn according to Westerheide and Boss (1999). Interactions among the DNA binding factors, CIITA, general transcription factors (TAFs), and HATs are indicated. The transcriptional elongation factor, pTEFb, is also shown.

(B) Negative and positive regulation of MHC class II and CIITA. Positive or negative regulatory processes typically target CIITA transcription or protein, which then targets MHC class II expression. (Left) Positive regulators include IFN-γ, IL-4, LPS, and IL-1. The pathway for IFN-γ is best delineated, involving the induction of P3 through STAT1, and the induction of P4 through IRF-1, IRF-2, and STAT1. (Right) Negative regulators include TGF-β, IL-10, IFN-β, and nitric oxide. Suppression of CIITA P4 expression by TGF-β requires SMAD3, while suppression of CIITA function by IFN-β requires ISGF3. It is unclear which promoters are affected by IL-10, NO, and IFN-β. PGE inhibits CIITA protein function by PKA-mediated phosphorylation. In addition, epigenetic events such as histone deacetylation and DNA methylation, and developmentally expressed molecules such as BLIMP1, negatively regulate CIITA production. HDACs also controls MHC class II expression through a CIITA-independent pathway. Dark blue ovals represent cell nuclei.

(Peretti et al., 2001). In addition to its specificity for the X1 element, the RFX complex also binds the S/W element (Jabrane-Ferrat et al., 1996). Finally, a protein which binds to the X2 box was purified and then identified as CREB (Moreno et al., 1999). CREB is bound to the MHC class II promoter as shown by the chromatin immunoprecipitation (ChIP) assay. It also interacts with CIITA and RFX, forming a final anchor in the large transcriptosome complex.

The DNA binding proteins mentioned above are all constitutively expressed, which cannot explain the cell-specific, cytokine-induced, and developmentally regulated expression of MHC class II genes. The isolation of the MHC class II transactivator gene (*MHC2TA*) solved much of this problem, and remains one of the seminal discoveries in the field (Steimle et al., 1993). *MHC2TA* was identified by complementation cloning of the RJ2.2.5 cell line (see Table 1) using an EBV-based episomal cDNA library. Complementation of RJ2.2.5 with a vector containing *MHC2TA*, encoding CIITA, resulted in the expression of surface class II antigens. Because CIITA does not bind DNA, it is an authentic transcriptional coactivator, defined as a transcription factor that mediates its function through interaction with other proteins. Long thought to be unique, CIITA is now recognized as a founding member of the NACHT protein family, which share several domains, including NTPase and Walker A and B motifs, and have roles in inflammatory responses and apoptosis (Koonin and Aravind, 2000).

Expression and Regulation of CIITA

Unlike RFX and NF-Y, CIITA exhibits cell-specific, cytokine-inducible, and differentiation-specific expression that precisely parallels that of MHC class II synthesis. Likewise, class II$^+$ cells, such as B cells, monocytes, dendritic cells, and human activated T cells, express CIITA (Harton and Ting, 2000; Reith and Mach, 2001). Additionally, an in vivo study has shown that the expression of CIITA under inflammatory transplantation conditions parallels the expression of MHC class II (Sims and Halloran, 1999). CIITA transcription is upregulated by IFN-γ, LPS, and IL-4, and is downregulated by IFN-β, IL-10, nitric oxide, and TGFβ (Figure 2B) (Harton and Ting, 2000; Reith and Mach, 2001). The induction by IFN-γ and downregulation by TGFβ are best worked out; the former is mediated by IRF-1, IRF-2, USF-1, and STAT1, and the latter by Smad3 (Dong et al., 2001; Xi et al., 1999). The regulation of CIITA expression occurs primarily at the transcriptional level; an exception is the suppression of CIITA activity by prostaglandins in myeloid-monocytic cells when PGE-induced, cAMP-dependent PKA causes the phosphorylation of CIITA (Li et al., 2001). Developmentally, CIITA suppression also occurs when B cells differentiate into plasma cells; this is attributed partly to the BLIMP-1/PRD transcription factor expressed in plasma cells (Ghosh et al., 2001; Piskurich et al., 2000). Another level of regulation is at the epigenetic level, where DNA methylation of the CIITA promoter suppresses its expression in trophoblasts (Morris et al., 2000), while inhibitors of histone deacetylases enhance MHC class II expression through both CIITA-dependent and -independent pathways (Magner et al., 2000).

Cell-Specific Promoters and Isoforms of CIITA

An area of research that has received much attention is the finding that *MHC2TA* contains multiple promoters

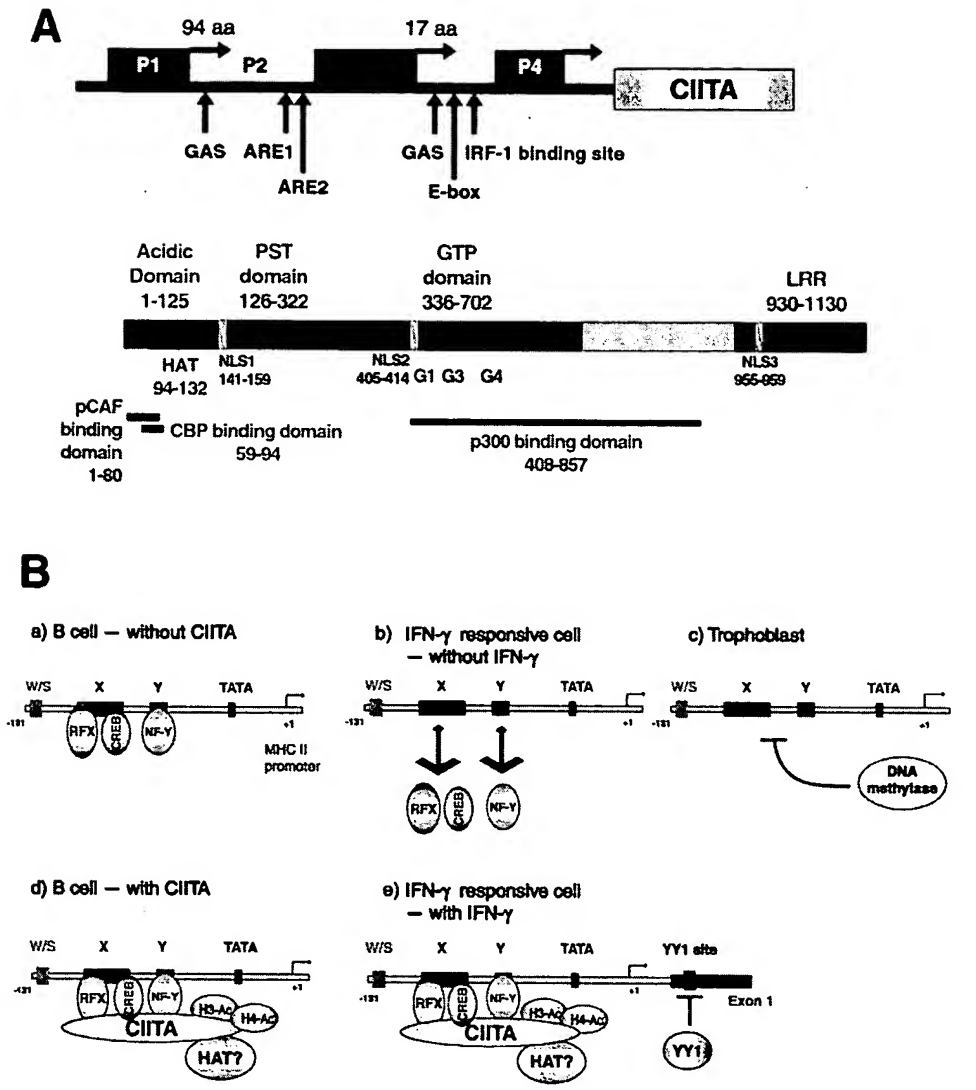

Figure 3. Regulation, Structure, and Function of CIITA

(A) Promoters of *MHC2TA* and structure of CIITA. The promoter of the *MHC2TA* (top) shows the different regulatory elements (arrows) found in the various promoters. A distal GAS site which responds to STAT1 is shown, although the precise location is unclear. The structure of CIITA protein (bottom) shows the different domains described in the text.

(B) Different states of the MHC class II transcriptosome. (a) In B cells, CIITA is not required for occupancy of the promoter by RFX and NF-Y. (b) In IFN-γ responsive cells, the promoter is bare or weakly bound in the absence of CIITA. (c) In cells which are not known to express class II antigens, such as trophoblasts, the promoter is silenced by DNA methylation. (d) The presence of CIITA in B cells causes H3 and H4 acetylation. Whether this is through tethered HATs, or through CIITA's intrinsic HAT activity, or both, is unclear. (e) Introduction of CIITA or induction by IFN-γ causes promoter occupancy and H3/H4 acetylation in IFN-γ responsive cells. YY1, a known HDAC, blocks IFN-γ-induced class II mRNA expression through a YY1 binding site found in the first exon.

directing the synthesis of at least three different 5′ coding sequences (Muhlethaler-Mottet et al., 1997). This indicates that CIITA function is regulated in a complex fashion, controlled by both cell-specific promoters as well as cell-specific isoforms. Although the precise expression of these isoforms is still undergoing revision, the current understanding is summarized as follows (Figure 3A, top). Promoter 1 (referred to as P1) is used in dendritic cells and leads predominantly to the expression of the longest isoform of 132 kDa (Landmann et al., 2001). It is extinguished when immature dendritic cells differentiate into a mature phenotype, and the extinction can be reversed by an inhibitor of histone deacetylases (HDACs). The extra 94 aa found in this isoform, as compared to the P3 isoform, encodes a caspase activation and recruiting domain (CARD) (Nickerson et al., 2001). This isoform is quantitatively more efficient than the P3 isoform in activating a DR promoter, perhaps explaining the higher concentration of class II molecules on dendritic cells. P2 is not clearly defined. P3 causes the generation of a 124 kDa isoform which is constitutively expressed in B cells. A short promoter region of 200+ bp for P3 is required for expression in B cells; two in vivo footprints in this region correspond

to two functional promoter elements, ARE-1 (a TEF-like element) and ARE-2 (Ghosh et al., 1999). On the other hand, a long promoter for P3 that extends 6 kb upstream is required for activation in IFN-γ-treated macrophage/monocytic lines in a STAT1-dependent fashion, and it is also abundantly expressed in IFN-γ activated melanomas and glioblastomas (Piskurich et al. 1999, Deffrennes et al., 2001; Goodwin et al., 2001). It is also expressed by immature dendritic cells, and is similarly silenced upon maturation (Landmann et al., 2001). P4, originally suggested to represent the primary IFN-γ-inducible promoter, is responsive to a combination of IRF-1, STAT1, and USF transcription factors, and produces the shortest isoform of 121 kDa. However, a targeted deletion of this promoter region in mice showed that it is crucial for the expression of MHC class II in nonhematopoietic cells, including cortical thymic epithelial cells, but not for hematopoietic cells (Waldburger et al., 2001). The most straightforward explanation is that this form is normally not required for CIITA expression in hematopoietic cells, although there is a possibility that other promoter-isoform pairs may have compensated for the loss of this form in hematopoietic tissues.

In summary, this complex array of *MHC2TA* promoters suggests that the fine-tuning of MHC class II expression must be crucial to assure a balance of selective immunity to foreign pathogens/antigens and tolerance to self-antigen. The timing and regulation of CIITA isoforms in distinct tissues have to be tightly regulated to assure proper immune function. The complexity of environmental signals, developmental programs, and cell-specific information must be interpreted accurately at the level of *MHC2TA* regulation to achieve appropriate MHC class II expression.

Structure and Function of CIITA

Structure-function analyses of CIITA have revealed the presence of both conventional domains expected of transcriptional activators, as well as unorthodox motifs (see Harton and Ting, 2000) (Figure 3A, bottom). The acidic domain at the N terminus (residues 1–125) is required for transactivation function, which may be achieved by providing an interaction surface for the histone acetylase CBP and RFXANK (Fontes et al., 1999; Kretsovali, 1998; Zhu et al., 2000). This segment also contains an intrinsic histone acetyltransferase (HAT) domain (Raval et al., 2001) (see below). A proline-serine-threonine-rich domain (residues 126–336) that contains multiple potential phosphorylation sites then follows. The midsection of the protein contains an unusual sequence for a transcription coactivator, the GTP binding domain (residues 337–702), that is involved in protein self-association and is important in nuclear import (Kretsovali et al., 2001; Linhoff et al., 2001; Sisk et al., 2001). Finally, a leucine-rich region (LRR) that also affects nuclear translocation and the self-association process resides at the C terminus. It associates with a 33 kDa protein; however, the identity and significance of this protein are presently unclear (Hake et al., 2000). Scattered amidst the protein are three nuclear translocation sequences, including two conventional nuclear localization sequences (NLS) and a bipartite NLS (Cressman et al., 1999, 2001; Kretsovali et al., 2001). An emerging theme is the shuttling of CIITA in and out of the nucleus, as evidenced by its sensitivity to the nuclear export inhibitor, leptomycin B, and by the identification of two sequences that are similar to nuclear export motifs which interact with the nuclear export protein CRM1.

Protein Complex Formation and Transcriptosome Assembly

Extensive protein-protein interactions involving all of the players described above occur across the MHC class II promoter to form an active and more stabilized transcriptosome (Figure 2A). Several reports have demonstrated interactions among peptides that bind to the X elements and NF-Y, and binding at these sites in an in vitro gel shift assay has a mutually enhancing effect on protein-DNA interactions (Harton and Ting, 2000; Reith and Mach, 2001). Analysis of the X2 binding protein, CREB, shows that it also interacts with RFX to form a stable complex. These interactions are in agreement with the analysis of in vivo or genomic footprint analysis which allows the visualization of protein-DNA interactions in intact cells. These latter studies show that the in vivo binding of transcription factors to X1 and Y is interdependent, while occupancy of X2 is dependent on binding of both X1 and Y. A generally accepted working model is that NF-Y, RFX, and CREB all interact, and this interaction likely promotes the formation of a stable transcriptosome complex.

From its discovery, it was presumed that CIITA must interact with the DNA binding transcription factors, since it is not a DNA binding protein. Indeed, several groups have used different approaches to reveal such interactions. CIITA interacts with NF-YB and NF-YC, but only weakly with NF-YA; it also interacts with RFXANK/RFXB and RFX5. In addition, an insightful in vivo approach, namely the ChIP assay, used to examine protein components of a transcriptosome, has begun to reveal important information. This procedure utilizes antibody directed at a component of the transcriptosome to pull down interacting proteins, and hence their respective cognate DNA sequences. A ChIP analysis has shown that CIITA coprecipitates DNA sequences that correspond to the X and Y elements, and hence CIITA directly or indirectly interacts with X and Y binding proteins (Masternak et al., 2000).

In addition to the specific players described above, the MHC class II transcriptosome has other interacting partners. By yeast two-hybrid analysis, CIITA interacts with the basal transcription factor $TAF_{II}32$, a component of TFIID, and indirect evidence also indicates the involvement of $TAF_{II}250$ in CIITA-mediated transactivation. CIITA has also been observed to promote transcriptional elongation, presumably through its interaction with cyclin T1, which together with CDK9 forms the positive transcription elongation factor b (P-TEFb) (Kanazawa et al., 2000). Aside from the interaction of CIITA with basal transcription factors, NF-Y has been shown to recruit the TFIID complex and to enhance the affinity of holo-TFIID for a MHC class II promoter through interactions with a number of TAFs (Mantovani, 1999). Additionally, NF-Y also interacts with histones H3 and H4 as well as the HAT p300 (Caretti et al., 1999). Notably, CBP, a homolog of p300, is a CREB binding protein, and CREB

binds X2. Whether all these associations occur at the chromatin level at the MHC class II promoter is an important area of investigation.

Chromatin Modification

One of the hallmarks of gene expression is the alteration of chromatin accessibility and DNA methylation status. Earlier studies of the chromatin structure of MHC class II promoters utilized the genomic footprinting approach to show that different subgroups of BLS-derived B cell lines differ in the in vivo occupancy of their promoters by DNA binding factors (Figure 3B). In cells lacking RFX, all MHC class II promoters are bare (i.e., in vivo footprints are lacking), while in cells lacking CIITA, the promoters are occupied normally (Kara and Glimcher, 1991). This would suggest that RFX is crucial for promoter accessibility, while CIITA is not. Indeed, in an RFX-defective, IFN-γ responsive cell line (G1B), the promoter is also bare despite IFN-γ treatment, which verifies the important role of the RFX protein in promoter assembly in both constitutive and inducible model systems (Brickey et al., 1999). In contrast, the role of CIITA in promoter occupancy in B cells and in an IFN-γ inducible system is in disagreement. In an IFN-γ inducible system, where CIITA is not expressed, or expressed at a minimal level prior to cytokine treatment, MHC class II promoters are minimally occupied; the addition of IFN-γ causes accessibility of the promoter in a time-dependent fashion (Harton and Ting, 2000). This would suggest that CIITA is the crucial factor for promoter accessibility. Indeed, when CIITA was transfected into MHC class II-negative cells, the nonclassical *DM* as well as *Ii* promoters became bound by factors. The precise reason for the different dependency on CIITA between B cells and IFN-γ responsive cells remains unresolved. One reasonable model is that the ubiquitous factors such as RFX and/or NF-Y may be expressed at higher concentrations in B cells, which obviates the absolute need for CIITA to stabilize the transcriptsome. This possibility was suggested by a report which noted higher levels of RFX in B cell lines than in IFN-γ responsive lines (Moreno et al., 1997). Despite these differences, CIITA remains pivotal for the transcription of MHC class II genes in both B cells and IFN-γ responsive cells.

How chromatin accessibility occurs over MHC class II promoters is an increasing focus of study. Both CIITA and NF-Y can interact with HATs, the former with p300, CBP, and pCAF, the latter with p300 (Fontes et al., 1999; Kretsovali, 1998). Cotransfection with CIITA and CBP, pCAF, or p300 can lead to increased activation of a MHC class II promoter-reporter construct. Despite this enhancement, the HAT domains of CBP and pCAF are dispensable for this activation (Harton et al., 2001). This would agree with the findings of two reports. The first shows that CIITA has intrinsic HAT activity, and thus may render other HATs dispensable (Raval et al., 2001). CIITA's HAT domain shares sequence homology with CBP and can substitute for the HAT function of $TAF_{II}250$. A second study found that two acetylated lysine residues within CIITA are important for nuclear import and that CBP and CAF may serve an alternate function in facilitating CIITA import into the nucleus (Spilianakis et al., 2000). More detailed analysis of the relevance of CBP/p300/CAF in promoter assembly will require analysis of the endogenous promoter under most physiologic conditions. A recent study has begun to address this issue by employing the ChIP assay in conjunction with real time PCR to assess the relationship between histone acetylation and CIITA occupancy (Beresford and Boss, 2001). They found that the presence of CIITA correlated with the acetylation of H3 and H4 at the endogenous MHC class II promoter in both B cells and an IFN-γ-inducible cell line. This result should be interpreted in light of the in vivo footprint analysis described above where binding of factors to endogenous MHC class II promoters in B cells does not require CIITA, while CIITA is required in IFN-γ responsive cells. This new study shows that regardless of cell type, CIITA is essential for proper histone acetylation in both cell groups. These results distinguish between factor binding to promoters and histone acetylation, and conclude that the former can occur without the latter, although transactivation does not occur in the absence of CIITA or histone acetylation (Figure 3B).

Finally, the role of histone deacetylase complexes (HDACs) in MHC class II gene control has begun to emerge. The general HDAC inhibitor, TSA, can rescue class II expression in tumor cells and mature dendritic cells where CIITA promoters are silenced (Landmann et al., 2001; Magner et al., 2000). Similarly, in a system where class II expression is silenced in the absence of the retinoblastoma protein (Rb), the addition of TSA restored expression and YY1 was identified as one of the HDACs (Osborne et al., 2001). Prior to the inhibition of HDAC, the promoter was found to exist in a nucleosome-free, DNase hypersensitive configuration, indicating that HDAC can exert its effect despite the establishment of a chromatin environment favorable for transcription initiation, and presumably with proteins bound to the promoter. This again suggests that modification of the acetylation state of histones and binding of proteins to the promoter occur independently.

Specificity of CIITA and RFX for MHC Class II Gene Expression

Two questions have been raised regarding the specificity of CIITA and RFX: (1) Do these factors control other genes? (2) Does MHC class II expression persist when either of these factors is missing? The first question has to be considered against the backdrop that the specificity of transcription factors is frequently invoked when they are first described, but this specificity fails to withstand more extensive investigation. CIITA has largely escaped this fate, with rare exceptions; many of its effects are specific for MHC class II molecules or its associated proteins. This is supported by representation difference analysis (RDA), which shows that most, if not all, of the genes induced by CIITA are within the class II pathway (Taxman et al., 2000). The control of the *DOA* and *DOB* genes by CIITA is less straightforward. Two reports showed common findings in the regulation of these two genes: the first used the aforementioned RDA method (Taxman et al., 2000), while the second used a chip array analysis (Nagarajan et al., 2002). Both found that in B cells, *DOA* is dependent on CIITA for expression, while *DOB* is expressed even in cells lacking CIITA.

Additionally, *DOA* and *DOB* are dependent on RFX for gene expression. However, the array analysis accompanied by real-time PCR showed a 2-fold enhancement of DOB in the presence of CIITA, which was not detected by RDA. This is reasonable, as the latter is best for detecting all-or-none differences, while array analysis detects more quantitative differences. The array paper also showed that the introduction of CIITA into an IFN-γ-inducible system does not greatly enhance *DOB* expression, in contrast to the great enhancement of *DOA* and *DRA* by CIITA. This agrees with an earlier finding that IFN-γ likewise significantly induces *DOA* but not *DOB* (Tonnelle et al., 1985). It is reasonable to conclude that *DOB* is less affected by CIITA than other class II MHC genes, although an effect can be detected, and that a CIITA-independent pathway exists for its expression. To further complicate the picture, CIITA$^{-/-}$ mice retain expression of both *H-2OA* and *B* as observed by RT-PCR. Whether this represents differences between humans versus mice, in vitro cell lines versus in vivo primary tissues, or simply RT-PCR versus real-time PCR is unclear.

CIITA is known to control a few genes other than MHC class II, although none are as strongly induced as class II genes. Class I MHC promoter and antigen expression is enhanced by CIITA in both human and murine lines (reviewed in van den Elsen and Gobin, 1999). CIITA regulation of class I is mapped to a region that has S/W, X, and Y homologs; ChIP analysis also shows the presence of CIITA at the endogenous β2*M* promoter, which likewise contains X and Y elements (Masternak et al., 2000; Riegert et al., 1996). A reduction of class I MHC has been observed in human Group A BLS patients but not in CIITA$^{-/-}$ mice. The reason for this discrepancy is unclear. In addition to class I MHC, a handful of genes have been found to be downregulated by the presence of CIITA, including *IL-4, fas*, and collagen (Gourley and Chang, 2001; Sisk et al., 2000; Zhu and Ting, 2001). In all these cases, CIITA mediates suppression by squelching general HATs. One of these studies compared the defective G3A cell line, in which IFN-γ induction of CIITA is suboptimal, to its normal parent, thus allowing the investigation of endogenous CIITA in gene suppression (Zhu and Ting, 2001). The observation was made that CIITA induction by IFN-γ can lead to the suppression of genes that are known suppressive targets of this cytokine. Hence, CIITA represents a dual-function factor, both as a strong inducer of immune response genes, and a repressor of general histone acetyltransferases for certain genes that may not be of immediate use during an IFN-γ response.

The second question regarding CIITA and RFX is whether they are indispensable for MHC class II expression. Mice lacking functional CIITA or RFX5 have been used to address this issue. CIITA$^{-/-}$ mice are largely devoid of class II, although some residual expression remains. Analyses of two CIITA$^{-/-}$ mice revealed substantial (20%) residual class II expression on dendritic cells limited to the s.c. lymph nodes detected by immunohistochemistry (Williams, 1998). The analysis of a third CIITA$^{-/-}$ strain revealed very low levels of class II mRNA only detected by RT-PCR, but not Northern hybridization, in the lymph nodes and spleen (Itoh-Lindstrom et al., 1999). A more recent study utilized one of the CIITA$^{-/-}$ mice used in the first study and found that class II expression on dendritic cells is reduced 99% as assessed by real-time PCR, and agrees with the notion of CIITA serving as a master regulator (Landmann et al., 2001).

The analysis of RFX5$^{-/-}$ mice showed class II expression on thymic medulla, mature dendritic cells, and activated B cells, but not B cells or IFN-γ activated macrophages (Clausen et al., 1998). Despite this residual expression, both CIITA$^{-/-}$ and RFX5$^{-/-}$ mice show severe immunodeficiency and CD4^{+} T cell defects, replicating the findings in humans. However, the CIITA$^{-/-}$ mice do respond differently from mice lacking Aβ, which are generally considered to be class II defective. In the nonobese diabetes (NOD) model, CIITA$^{-/-}$ NOD mice have pancreatic cellular infiltrates, while Aβ$^{-/-}$ mice do not (Mora et al., 1999), yet neither display symptoms of diabetes. Whether this difference between the two strains can be attributed to residual class II expression, to other class II-associated genes (*Ii, M, O*), or to nonassociated genes that are selectively controlled by CIITA remains to be determined.

Disease and Physiologic Relevance

The MHC class II loci are associated with more diseases than any other region of the genome of equivalent size. Class II-related autoimmune conditions are suspected to be due to a failure of tolerance. Expression of class II genes must be tightly and subtly controlled to ensure appropriate vigorous responses to pathogens while minimizing collateral damage to host tissues.

Considering that CIITA is a master regulator of MHC class II gene transcription, it represents an ideal target for pathogens to evade the immune system. Indeed, a variety of pathogens (*cytomegalovirus* (CMV), *Mycobacterium bovis, Chlamydia, varicella-zoster* virus, parainfluenza virus, and Epstein Barr virus (EBV)) have evolved several pathways to alter the expression of CIITA (reviewed in Harton and Ting, 2000; Reith and Mach, 2001; Accolla et al., 2001; see also Gao et al., 2001; Morrison et al., 2001). Furthermore, HIV-1 infection also suppresses class II expression by interfering with both CIITA and NF-YA functions (Rakoff-Nahoum et al., 2001). One common feature is that most of the pathways utilized by pathogens affect a more general target upstream of CIITA expression, such as the Jak/Stat pathway, the USF-1 transcription factor, IFN-γ receptor expression, and other cytokines which can affect class II expression; thus, the effects are far-reaching beyond MHC class II regulation. Additionally, it was found that statin, a drug used in the treatment of heart disease to control lipid levels, also decreases CIITA expression by interfering with P4 function (Kwak et al., 2000). This raises the possibility that these drugs might be useful to regulate immune activation involving the hyperexpression of class II antigens, as in autoimmune or autoinflammatory disorders.

Both class II MHC and CIITA promoters exhibit polymorphisms that may be relevant to disease. This is not surprising, since the expression level or control dynamics of a class II molecule could help to modulate the immune response (Baumgart et al., 1998). It may influence cytokine profiles or the ratio of Th1 to Th2 re-

sponses, for example. Conversely, promoter variation could influence susceptibility to disease independent of coding region variation. A number of variant nucleotides have been described in promoter regions upstream of class II genes. Some of these are in canonical sequences identified as binding elements for transcription factors, and in a few cases the changes have been shown to affect transcription in reporter assays (Andersen et al., 1991). In addition to promoter variation, intergenic regions several kb upstream of class II loci may exhibit marked variation. The *DQB1* gene is marked by variation in the presence or absence of retroviral LTRs that could in principle influence expression levels (Kambhu et al., 1990). Finally, more recent reports have shown polymorphisms in the CIITA promoter and 3′ untranslated region, although the significance to disease is not clear (Rasmussen et al., 2001).

The utilization of CIITA as a means to enhance cancer immunotherapy has also been investigated. There is some disagreement concerning the ability of CIITA-transfected cells to present exogenous antigens (Sartoris et al., 1998; Siegrist et al., 1995), and this may simply vary with the cell line examined. However, two studies which directly examined antitumor activity of different CIITA-transfected tumors have failed to show an efficacy of CIITA in enhancing tumor control, despite the presence of costimulatory molecules such as CD80 and CD86 (Armstrong et al., 1997; Martin et al., 1999). These studies showed a correlation with the poor ability of CIITA-induced class II complexes to present antigens targeted through the endogenous pathway, which may represent the route traveled by many tumor antigens. Overcoming such issues will be necessary to exploit the utilization of CIITA as a potential therapeutic in tumor control. As an alternate strategy, an adenoviral construct containing *MHC2TA* has been introduced into dendritic cells with enhanced immunostimulatory effects (Marten et al., 2001), which warrants the testing of this strategy in animal models.

Summary

The MHC class II system has evolved into a complex array of surface molecules which are required to bind peptides of a wide ranging number of pathogens. The classical and nonclassical MHC class II genes, in addition to Ii, have one predominant function: presenting antigenic peptides and activating CD4 T cells in hematopoietic as well as extrahematopoietic tissues. The molecular control of these molecules is highly complex and intricate. Strict coordination of MHC class II gene control has been implemented to deal efficiently and appropriately with foreign antigens, while minimizing autoreactive responses against self-antigens. A master transcriptional regulator, CIITA, which is highly specific in its function, controls the expression of this entire class of genes. This is performed by coordinating the functions of DNA binding proteins and histone acetylases to modify local chromatin and promoter accessibility. DNA methylases and HDAC are also involved, and these are likely to represent areas of intense research focus. Future endeavors to harness this knowledge to modulate disease outcome, whether infectious diseases, autoimmunity, or cancer therapy, should be of tremendous interest and potential.

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Combinations of dominant-negative class II transactivator, p300 or CDK9 proteins block the expression of MHC II genes

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Abstract

The class II transactivator (CIITA) regulates not only the transcription of HLA-DR, -DQ, -DP, but also invariant chain, DMA and DMB genes. A hybrid mutant CIITA protein, which contained residues from positions 302 to 1130 in CIITA fused to the enhanced green fluorescent protein (EdCIITA), inhibited the function of the wild-type protein. EdCIITA extinguished the inducible and constitutive expression of MHC II genes in epithelial cells treated with IFN-γ and B lymphoblastoid cells respectively. Also, it blocked T cell activation by superantigen. This inhibition correlated with the localization of EdCIITA but not CIITA in the cytoplasm of cells. However, when EdCIITA was co-expressed with a dominant-negative form of the nucleoporin Nup214/CAN, it also accumulated in the nucleus. These data suggest that EdCIITA not only competes with the wild-type protein for the binding to MHC II promoters but sequesters a critical co-factor of CIITA in the cytoplasm. CIITA also recruits the histone acetyltransferase cAMP responsive element binding protein (CREB) binding protein and positive transcription elongation factor b (p-TEFb) for the transcription of MHC II genes. Dominant-negative p300 (DNp300) or CDK9 (DNCDK9) proteins inhibited the function of CIITA and of the DRA promoter. Thus, combinations of EdCIITA and DNp300 and/or DNCDK9 proteins extinguished the transcription of MHC II genes. They might become useful for future genetic therapeutic approaches in organ transplantation and autoimmune diseases.

Introduction

MHC II molecules present foreign antigens to T_h cells and direct the subsequent differentiation of B cells. Their congenital absence leads to a severe combined immunodeficiency called the type II bare lymphocyte syndrome (BLS) (1). Using B lymphoblastoid cell lines and transient heterokaryon fusions, five complementation groups (A–E) of BLS were characterized. Whereas group E appears to affect a locus organizing region (2), the expression of all MHC II genes is extinguished in groups A–D. They contain mutations in genes that code for the class II transactivator (CIITA) and regulatory factor that binds to the X box (RFX), which is composed of three subunits, RFX-5, RFX-AP and RFX-ANK/B. In BLS-2 cells (group A), the CIITA gene has a short in-frame deletion which includes one of its nuclear localization signals (NLS) from positions 940 to 963 (3,4). Although CIITA contains an additional putative bipartite nuclear targeting sequence from positions 144 to 161 (5), both of which are conserved between human and murine CIITA proteins (6), this mutant CIITA protein is found in the cytoplasm and has no activity (4).

In the conserved upstream sequences (CUS) of MHC II promoters, RFX recognizes S and X boxes (7). Other proteins, which include the cAMP responsive element binding protein (CREB), nuclear factor Y (NF-Y: NF-YA, -YB and -YC) and octamer binding factor, bind to the downstream X2, Y and octamer sequences (8,9). They are critical for IFN-γ-inducible and constitutive expression of MHC II genes. CIITA neither binds directly to DNA nor appears in electrophoretic mobility shift assays (EMSA) with proteins assembled on CUS (3). Nevertheless, subunits of RFX, NF-Y and CREB have been reported to bind to CIITA in cells (10–12). In one report, this binding depended on p33, which is an unknown protein that binds to the C-terminal leucine-rich repeats of CIITA (13).

CIITA attracts several transcription factors to its N-terminal activation domain. These include the TATA-box binding protein

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associated factors TAFII32 and TAFII70, histone acetyltransferase CREB binding protein (CBP/p300) and cyclin T1, which forms the positive transcription elongation factor b (P-TEFb) (14–20). Thus, the N-terminal region of CIITA integrates steps for the initiation and elongation of MHC II transcription.

Several mutant CIITA proteins function as dominant-negative proteins and inhibit the expression of MHC II genes in cells (5,21–25). To date, the best is a truncated CIITA protein, which lacks the N-terminal acidic and P/S/T-rich sequences (23). However, this dominant-negative CIITA protein could not extinguish the expression of MHC II genes (23). Moreover, its mechanism of action remained unexplained. Also, the dominant-negative forms of CBP (DNCBP) and CDK9 (DNCDK9 from P-TEFb) block the function of CIITA (15,18). They affect steps subsequent to the initiation of MHC II transcription.

In this report, we demonstrate that a mutant CIITA protein from positions 302 to 1130 (dCIITA) fused to the enhanced green fluorescence protein (EGFP: EdCIITA) could inhibit the transcription of MHC II genes better than dCIITA. Surprisingly, EdCIITA was found in the cytoplasm. Nevertheless, a dominant-negative nucleoporin Nup214/CAN protein (ΔCAN), which blocks nuclear export, retained EdCIITA in the nucleus, suggesting that EdCIITA can also sequester a critical co-factor of CIITA in the cytoplasm (26). In addition, dominant-negative forms of p300 (DNp300) and CDK9 (DNCDK9) inhibited the DRA promoter. Combinations of EdCIITA, DNp300 and DNCDK9 repressed the expression of MHC II genes synergistically. Thus, complementary strategies are more effective in extinguishing the expression of MHC II genes.

Methods

Cell culture

HeLa and COS cells were grown in DMEM supplemented with 10% FCS and antibiotics (100 U/ml of penicillin G/100 μg/ml of streptomycine sulfate) at 37°C in 5% CO_2. Jurkat and RM3 cells were grown in RPMI 1640 supplemented with 10% FCS and antibiotics described above at 37°C in 5% CO_2.

Plasmids and the constructs

Either wild-type CIITA (amino acids 1–1130) or dCIITA (amino acids 302–1130) genes were fused with EGFP using pEGFP-C1 vector (Clontech, Palo Alto, CA), termed pECIITA and pEdCIITA respectively. Also, HA-tagged wild-type CIITA and dCIITA was subcloned into the same backbone vector as a control, termed pCIITA and pdCIITA respectively. The HA-tag sequence, 5′-TACCCATACGATGTTCCAGATTACGCTGCT-3′, was inserted after first ATG of the N-terminus of CIITA cDNA in pCIITA and pECIITA. HA-tagged CIITA has similar activity with wild-type CIITA in the CAT assay (J. D. Fontes and B. M. Peterlin, unpublished data). All plasmids were regulated under the cytomegalovirus promoter. All plasmid constructs were sequenced and their expressions confirmed by Western blotting. The reporter gene, pDRASCAT, contains the DRA promoter linked with the CAT gene, which have been previously described (27). The empty plasmid vector was used as a stuffer DNA in all experiments. Plasmids coding for Tat, DNCDK9, DNp300 (a gift from K. Kelly), pcDNA3flagCIITA (a gift from J. P.-Y. Ting), pΔCAN (a gift from B. Cullen) and NF-AT–luciferase reporter gene have been described previously (18,22,26,28).

CAT assay, the analyses of the localization of EdCIITA, ECIITA and EGFP in the cells, and Western blotting

Effector gene, pCIITA, and reporter gene, pDRASCAT (0.25 or 0.4 μg each), were co-transfected with pdCIITA, pEdCIITA, pΔCAN or stuffer DNA (0.25–1.5 μg) in COS cells by lipofectamine (Life Technologies, Germantown, MD). COS cells (2×10^5) were incubated with 6 μl of lipofectamine with DNAs for 16 h. Cells were harvested and extracted with 200 μl of lysis buffer (0.25 M Tris–HCl, pH 7.5, 0.1% Triton X-100) 24 h after transfection. Lysates were inactivated at 65°C for 10 min, then mixed with 50 μl of counting buffer [0.2 μl of [^{3}H]acetyl-coenzyme A (Amersham Life Science, Chicago, IL), 5 μl of 8.3 mg/ml chloramphenicol, 5 μl of 0.25 M Tris–HCl, pH 7.5]. Radioactivities were counted by scintillation counter (Beckman Instruments, Fullerton, CA). For the analyses of the localization of EdCIITA, ECIITA or EGFP, each construct was transfected in COS cells with or without ΔCAN by lipofectamine as described above. The ratio of amounts of ΔCAN against each plasmid DNA was 1:3. The localization of EGFP fusion proteins was analyzed 48 h after transfection by a Nikon E-800 fluorescence microscope (Nikon, Melville, NY). For the expression of EdCIITA and dCIITA, DNA or the empty plasmid vector (1 μg each) were transfected into COS cells. Proteins were immunoprecipitated with a rabbit polyclonal antibody against the C-terminus of CIITA. Western blotting was performed with the same antibody or an anti-actin antibody (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA)

IFN-γ treatment

HeLa cells (1×10^7) were transfected with pEdCIITA (20 μg) or the empty plasmid DNA (20 μg) by electroporation (BioRad electroporator; 975 μF, 300 V). DNA-transfected HeLa cells (2×10^5) were cultured for 24 h and then the cells were incubated with human IFN-γ (1000 U/ml; Boehringer Mannheim, Indianapolis, IN) for 72 h. HeLa cells were harvested and stained with appropriate antibodies, and then analyzed by FACS (Becton Dickinson, San Jose, CA).

Electroporation and superantigen assay

RM3 cells (1×10^7) were transfected with pCIITA (20 μg) and either pEdCIITA or the empty plasmid DNA (20 μg each) by electroporation as described above. The cells were harvested 48 h after transfection and stained with appropriate antibodies for FACS analyses. For superantigen assay, the electroporation was performed 48 h prior to co-culture. On the other hand, NF-AT–luciferase reporter gene (40 μg) was transfected in Jurkat cells (1×10^7) by electroporation 24 h prior to co-culture. The transfected Jurkat cells were cultured with either the transfected or untransfected RM3 in the presence or absence of Staphylococcal enterotoxin D (SED; Toxin Technology, Sarasota, FL) for 16 h. Cells were lysed and luciferase assays were performed as described previously (18).

FACS analyses

Both transfected HeLa cells and RM3 cells were stained with anti-DR (clone L243), -DQ (clone SK10) or -DP (clone B7/21)

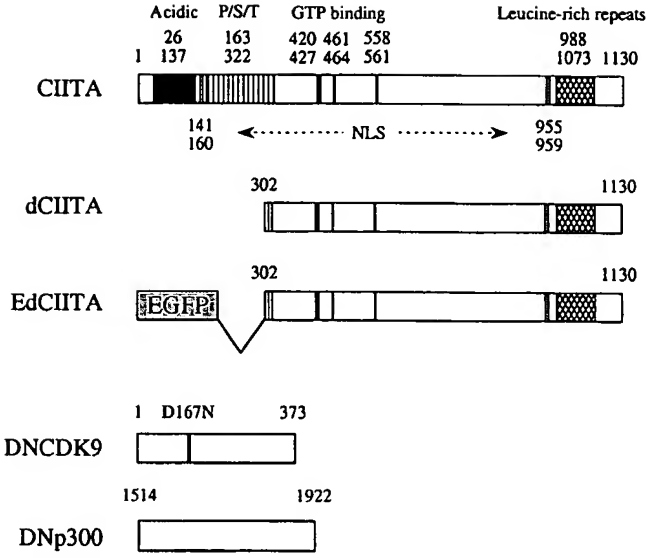

Fig. 1. The structure of CIITA (ECIITA), dCIITA, EdCIITA and dominant-negative effector proteins. CIITA contains 1130 residues, which can be grouped into five specific domains. From the N-terminus, they are the acidic activation domain (positions 26–137), a P/S/T-rich region (positions 163–322), three GTP binding consensus motifs (positions 420–427, 461–464 and 558–561), two NLS (positions 141–160 and 955–959) and the leucine-rich repeats (positions 988–1073). ECIITA contains the HA epitope tag (TPTDVDTAA) at its N-terminus, which is fused with the C-terminus of EGFP. The acidic and P/S/T-rich regions are deleted in dCIITA and EdCIITA (positions 302–1130). CBP and cyclin T1 from P-TEFb bind to the N-terminus of CIITA (positions 1–144 and 1–322 respectively). DNCDK9 contains an asparagines rather than aspartic acid at position 167, which inactivates the kinase activity of CDK9. DNp300 contains residues from positions 1514 to 1922 in p300, which blocks histone acetyltransferase activity.

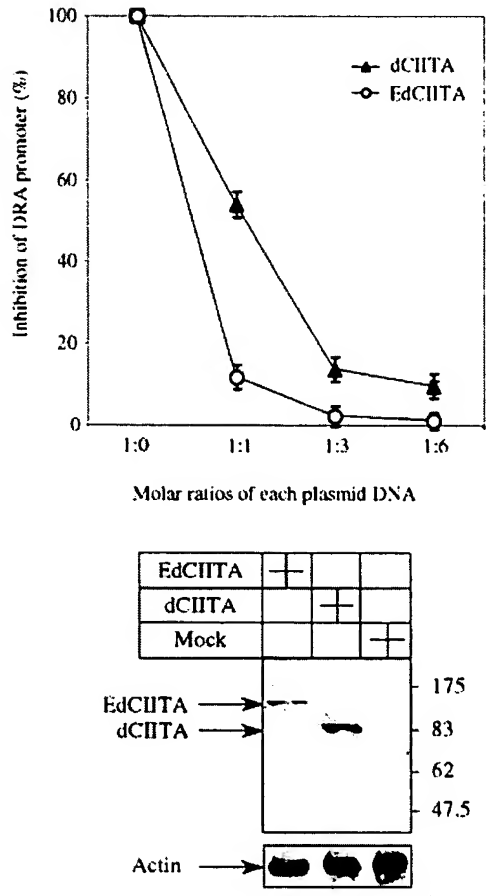

Fig. 2. dCIITA and EdCIITA inhibit the DRA promoter. dCIITA or EdCIITA were co-expressed with CIITA and DRA promoter, on pDRASCAT, in COS cells. 1:1, 1:3 and 1:6 represent molar ratios between CIITA and competitor plasmid DNA. The inhibition of the DRA promoter was calculated and compared to the control, where only CIITA was co-expressed with the plasmid target (%). CAT enzymatic assays were performed as described previously (27). Data represent the inhibition of EdCIITA (O) and dCIITA (▲) respectively. Error bars give SEM from three experiments performed in duplicate. Below the graph are presented expression levels of EdCIITA, dCIITA and actin, which were determined by Western blotting. Lysates were immunoprecipitated with the rabbit polyclonal anti-CIITA antibody and then blotted with the same antibody. Levels of actin were determined with an anti-actin antiserum.

antibodies in PBS with 5% BSA/0.1% sodium azide for 20 min. Secondary staining was performed with phycoerythrin-conjugated rat anti-mouse κ antibody (clone X36) (all antibodies were purchased from Becton Dickinson, Mountain View, CA). The expressions of MHC II proteins and EdCIITA were analyzed by FACS.

Results

EdCIITA prevents the activation of the DRA promoter by CIITA

To create an optimal dominant-negative CIITA protein, we relied on previously published work (5,21–25). dCIITA lacks the first 301 amino acids of CIITA, which include acidic (positions 26–137), bipartite nuclear targeting (positions 144–161) and P/S/T-rich sequences (positions 163–322) (Fig. 1). The wild-type CIITA and dCIITA proteins were fused in-frame to the C-terminus of EGFP, and termed ECIITA and EdCIITA respectively. Next, we compared effects of these proteins on the DRA promoter. dCIITA or EdCIITA were co-expressed with CIITA, and the plasmid target, pDRASCAT, in COS cells. All transcripts coding for our proteins were transcribed from the cytomegalovirus immediate-early promoter. The inhibition of the DRA promoter was observed in a dose-dependent manner for each combination of plasmids. At a 1:1 molar ratio of co-transfected plasmids, EdCIITA repressed the DRA promoter 10-fold, and extinguished it to background levels at 1:3 and 1:6 molar ratios (Fig. 2, open circles). dCIITA was much less potent at these concentrations and never blocked the DRA promoter completely (Fig. 2, solid triangles). Although only effects at 72 h are presented, identical results were obtained at shorter and longer times after transfection (data not presented). EdCIITA could function better than dCIITA because of its different composition (the addition of EGFP), greater stability or subcellular localization. We conclude that EdCIITA is a strong dominant-negative CIITA protein. Moreover,

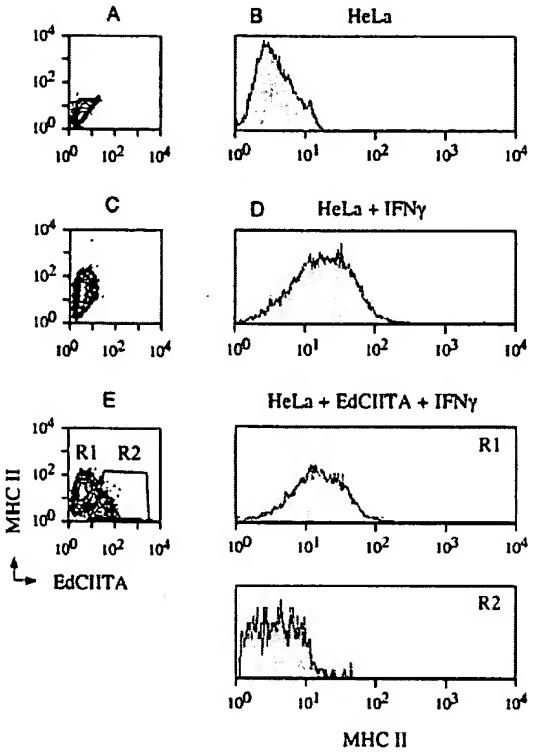

Fig. 3. IFN-γ induces the expression of MHC II genes in HeLa but not in HeLa cells that express EdCIITA. (A and B) HeLa cells were not treated with IFN-γ. (C and D) HeLa cells were treated with IFN-γ. (E and F) HeLa cells were transfected with EdCIITA and treated with IFN-γ. Except for (A) and (B), all cells were treated with human IFN-γ at the concentration of 1000 U/ml in the culture medium for 72 h after the transfection. Cells were stained with the anti-DP antibody and analyzed by FACS. Two-dimensional histograms (A, C and E) show the expression of DP and EdCIITA. Histograms in panels B, D, R1 and R2 show the expression of DP. R2 displays a gated region in (E) that contains cells, which expressed EdCIITA. R1 represents the remaining population of untransfected cells.

because it is linked to EGFP, its levels of expression can be monitored easily in cells.

IFN-γ fails to induce the expression of MHC II genes in HeLa cells expressing EdCIITA

The administration of IFN-γ activates the Jak/Stat signaling cascade and CIITA transcription (29,30). This induction is critical for the function of antigen-presenting cells. To confirm that EdCIITA could also block effects of IFN-γ, we expressed EdCIITA for 2 days in HeLa cells, which were subsequently treated with 1000 U/ml of IFN-γ for 3 days. Cells were stained with the antibody against DP and then analyzed by FACS (Fig. 3). The expression of EdCIITA was followed by green fluorescence and was abundant in ~30% of HeLa cells for the duration of the assay. In Fig. 3, they are visualized on the *x*-axis (Fig. 3E, R2). Cells in the R1 region did not express pEdCIITA and resembled untransfected cells (Fig. 3E). Importantly, the inhibition of DP was observed only in cells that expressed EdCIITA (Fig. 3, R2). These data were confirmed with antibodies against DR determinants (data not presented).

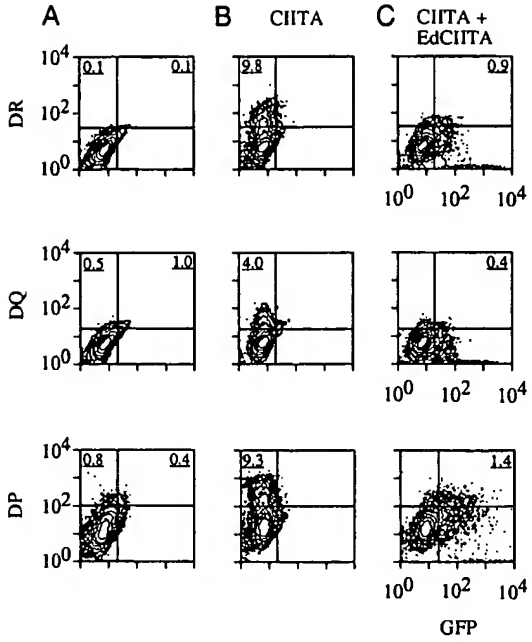

Fig. 4. EdCIITA inhibits the expression of MHC II genes in RM3 cells. RM3 cells expressed no exogenous protein (A), CIITA alone (B) or both CIITA and EdCIITA (C). Cells were cultured for 48 h after transfection and stained with anti-DR, -DQ or -DP antibodies respectively. The numbers in quadrants display the percentage of cells in each subcategory. Vertical bars indicate the levels of DR, DQ and DP determinants on the cell surface respectively. The horizontal bar represents levels of green fluorescence in RM3 cells (C).

Thus, EdCIITA also blocks the induction of MHC II genes by IFN-γ.

In the presence of EdCIITA, CIITA cannot restore the expression of MHC II genes in B lymphoblastoid RM3 cells

RM3 cells were established as a MHC II-deficient B cell line from Raji cells. However, the exogenous expression of CIITA in these cells leads to the synthesis of MHC II determinants to levels that are comparable to those on parental Raji cells. We chose RM3 because of the slow decay of pre-existing MHC II–peptide complexes on Raji and other antigen-presenting cells. As presented in Fig. 4(B), the introduction of CIITA restored high levels of expression of DR, DQ and DP determinants on RM3 cells. In sharp contrast, CIITA failed to rescue the expression of MHC II determinants in the presence of EdCIITA (Fig. 4C). This inhibition was from 9.8 to 0.9% of DR^+ cells, 9.3 to 1.4% of DP^+ cells and 4.0 to 0.4% of DQ^+ cells respectively (Fig. 4, *cf.* B and C). This inhibition was observed only in green fluorescent RM3 cells that expressed EdCIITA. These results were confirmed in RJ2.2.5 cells (data not presented). Thus, EdCIITA inhibits efficiently the expression of MHC II genes in B lymphoblastoid cells.

EdCIITA blocks the presentation of superantigen by MHC II determinants to T cells

EdCIITA blocked the expression of MHC II genes in kidney cells (COS) that expressed CIITA, IFN-γ-treated epithelial cells

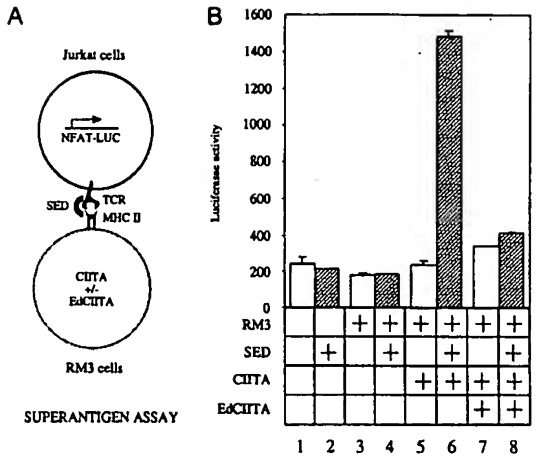

Fig. 5. RM3 cells that express EdCIITA fail to activate T cells via superantigen. (A) Schematic diagram describing the superantigen assay. (B) CIITA and EdCIITA, or CIITA alone were expressed in RM3 cells 2 days before co-culture with Jurkat T cells. NF-AT–luciferase reporter gene was transfected into Jurkat T cells 1 day before co-culture with RM3 cells. Open and shaded columns represent the absence and presence of SED respectively. Experiments are representative of three independent transfections, which were performed in duplicate. Error bars display SEM.

(HeLa) and B lymphoblastoid cells (RM3). Moreover, since Ii, DMA and DMB genes are also regulated by CIITA, EdCIITA should block antigen processing and presentation. To examine functional consequences of this blockade, we studied effects of EdCIITA on T cell activation via superantigen and MHC II determinants on B cells. The diagram of this experiment is presented in Fig. 5(A). Again, RM3 cells expressed CIITA with or without EdCIITA. Transfected cells were co-cultured with Jurkat cells, which contained the NF-AT–luciferase reporter gene, in the presence or absence of SED. MHC II complex:superantigen induces T cell signaling via the TCR and activates the NF-AT reporter gene in Jurkat cells. The expression of CIITA alone resulted in NF-AT activity only in the presence of SED (Fig. 5, lane 6). On the other hand, EdCIITA strongly inhibited our superantigen assay (Fig. 5, lane 8). Levels of inhibition correlated perfectly with the expression of MHC II genes and the inhibition of the DRA promoter (Fig. 2). Similar results were obtained when a plasmid target containing the NF-κB binding sites linked to luciferase was used (data not presented). Thus, EdCIITA blocks the expression and function of MHC II determinants.

Unlike CIITA, EdCIITA is localized in the cytoplasm

It is thought that the dominant-negative CIITA proteins, such as dCIITA, compete with CIITA for the binding to MHC II promoters in the nucleus of cells. To check the validity of this model, we examined the distribution of EdCIITA in COS cells. As presented in Fig. 6(A), ECIITA was found in the nucleus. In sharp contrast, EdCIITA was localized in the cytoplasm (Fig. 6B). EGFP alone was distributed throughout the cell (Fig. 6C). This result suggests that EdCIITA acts indirectly to block the function of CIITA. One possibility is that it sequesters a co-factor of CIITA in the cytoplasm.

Although mutant CIITA proteins lacking the C-terminal NLS and bearing mutations in GTP-binding domains are found in the cytoplasm, EdCIITA still contains both sequences. EdCIITA lacks only the putative N-terminal NLS (Fig. 1). Thus, EdCIITA should still enter the nucleus. Additionally, CIITA contains many leucines, especially near its C-terminus, some of which are spaced in a manner reminiscent of nuclear export signals (NES). Thus, it is possible that EdCIITA enters the nucleus but is rapidly exported to the cytoplasm. In the steady state, EdCIITA would then appear cytoplasmic. To examine directly whether these shuttling mechanisms in EdCIITA functioned, we co-expressed the dominant-negative nucleoporin ΔCAN, which blocks the function of NES (26). Indeed, when EdCIITA was co-expressed with ΔCAN, EdCIITA was localized in the nucleus (Fig. 6E). On the other hand, ECIITA and EGFP retained their previous subcellular distribution (Fig. 6D and F). We conclude that CIITA is a shuttling protein, that it contains functional NLS and NES, and that EdCIITA most likely competes for the binding to MHC II promoters in the nucleus as well as sequesters a co-factor of CIITA in the cytoplasm.

DNp300, DNCDK9 and Tat also inhibit CIITA and act synergistically with EdCIITA

Previously, we demonstrated that CIITA binds to CBP and cyclin T1 from P-TEFb to affect the chromatin conformation and elongation of transcription of MHC II genes. Dominant-negative forms of CBP or p300 (DNp300, Fig. 1) and CDK9 from P-TEFb (kinase dead CDK9, CDK9N167N, DNCDK, Fig. 1) could inhibit MHC II transcription. Since Tat, which is an essential transactivator of viral replication, bound to the same surface on cyclin T1 and blocked effects of CIITA, HIV-1 inhibits efficiently antigen processing and presentation by MHC II determinants. DNp300 and DNCDK9 are nuclear proteins that act at a post-assembly step, when DNA-bound proteins have already recruited CIITA. Thus, they should block the activity of the residual CIITA in B cells and antigen-presenting cells, and act synergistically with each other and EdCIITA.

Indeed, Tat and both dominant-negative proteins potentiated effects of EdCIITA in COS cells. Tat, DNp300 and DNCDK9 alone inhibited CIITA function on the DRA promoter (Fig. 7A, lanes 4, 6 and 8). They acted synergistically with small amounts of EdCIITA (Fig. 7A, lanes 5, 7 and 9). Moreover, the combination of EdCIITA, DNp300 and DNCDK9 extinguished the expression from the DRA promoter to background levels (Fig. 7B, lane 4). All dominant proteins were expressed to equivalent levels in these cells (data not presented). We conclude that EdCIITA, DNp300 and DNCDK9 act at different steps of MHC II transcription and thus synergistically to block the function of CIITA.

Discussion

In this study we demonstrated that EdCIITA inhibits efficiently the function of CIITA. EdCIITA diminished the expression of MHC II genes in HeLa cells, which were treated with IFN-γ, and in B lymphoblastoid RM3 cells. EdCIITA also inhibited T cell activation by superantigen. Surprisingly, at steady state, whereas CIITA was localized in the nucleus, EdCIITA was

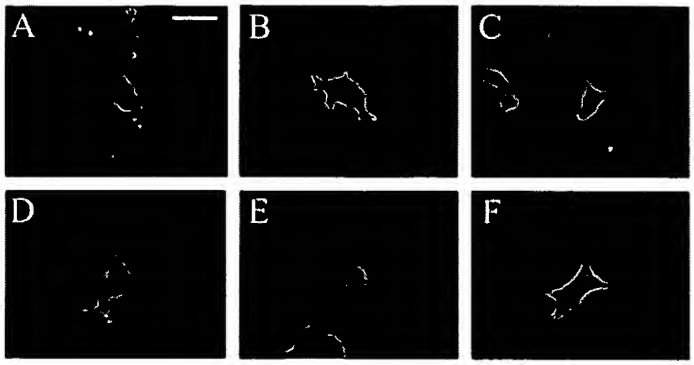

Fig. 6. At steady state, EdCIITA is found in the cytoplasm, but ΔCAN alters its localization to the nucleus. ECIITA, EdCIITA and EGFP were expressed with or without ΔCAN in COS cells. The expression of each protein was observed by fluorescence microscope 48 h after the transfection. (A–C) The expression of ECIITA, EdCIITA and EGFP respectively; (D–F) contain additionally ΔCAN but are otherwise the same as (A–C). Scale bar represents 50 μm in (A).

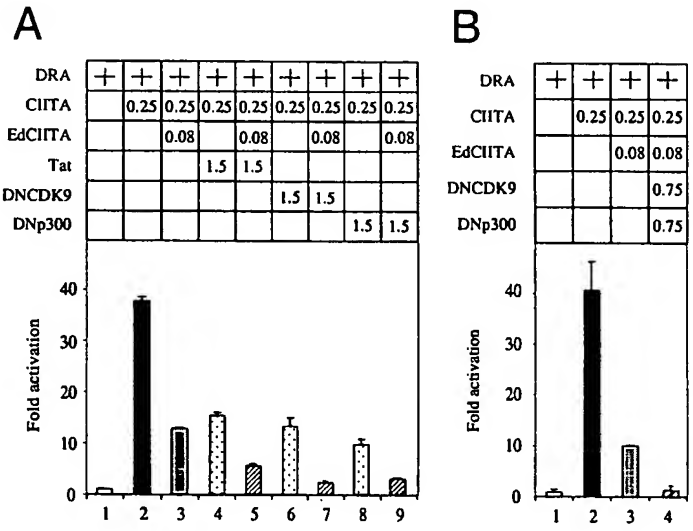

Fig. 7. Combinations of EdCIITA, DNp300 and DNCDK9 inhibit DRA promoter synergistically. (A) EdCIITA acts synergistically with Tat, DNp300 and DNCDK9 to inhibit the expression of MHC II genes. Combinations of the expression of each dominant-negative protein with EdCIITA are represented in the upper column. Values represent the amount of DNA (μg) in each transfection. Bar graphs represent the following: empty bar is the control (lane 1), black bar contains CIITA alone (lane 2) and gray bar represents EdCIITA alone (lane 3). The repression by each effector (lanes 4, 6 and 8) and that by combinations of EdCIITA with each effector (lanes 5, 7 and 9) are presented. (B) Together, all three dominant-negative proteins extinguish the expression of MHC II genes. Empty, black and gray bars represent the same combinations as in (A) (lanes 1–3). The combination of EdCIITA, DNCDK9 and DNp300 is presented in lane 4. Error bars give SEM.

found in the cytoplasm. However, EdCIITA is a shuttling protein. ΔCAN blocked the export of EdCIITA from the nucleus, which suggested that EdCIITA sequesters some co-factor of CIITA in the cytoplasm. DNp300, DNCDK9 or Tat inhibited the remaining CIITA in the nucleus. Since EdCIITA, DNp300, DNCDK9 or Tat affect different steps in the transcription of MHC II genes, they acted synergistically. We conclude that combinatorial approaches might offer the best hope of extinguishing the expression of MHC II genes in cells and in the organism.

This study used the most recent advances in our understanding of steps in the assembly and function of regulatory proteins on MHC II promoters. CREB, and subunits of NF-Y and RFX bind to DNA and to CIITA. However, this recruitment requires an additional protein of 33 kDa, which binds to the leucine-rich repeats in CIITA and possibly other co-factors. These other cellular proteins might explain the dichotomy between binding studies of individual components (CREB, CIITA, NF-Y and RFX) in cells and the inability of CIITA alone to bind to these proteins in EMSA. Additionally, CIITA binds to general transcription factors and CBP to initiate MHC II transcription. CIITA also binds to P-TEFb, which phosphorylates the C-terminal domain of RNA polymerase II to elongate MHC II transcription. Our dominant-negative strategies attacked these different steps.

First, EdCIITA linked dCIITA to EGFP. The latter addition

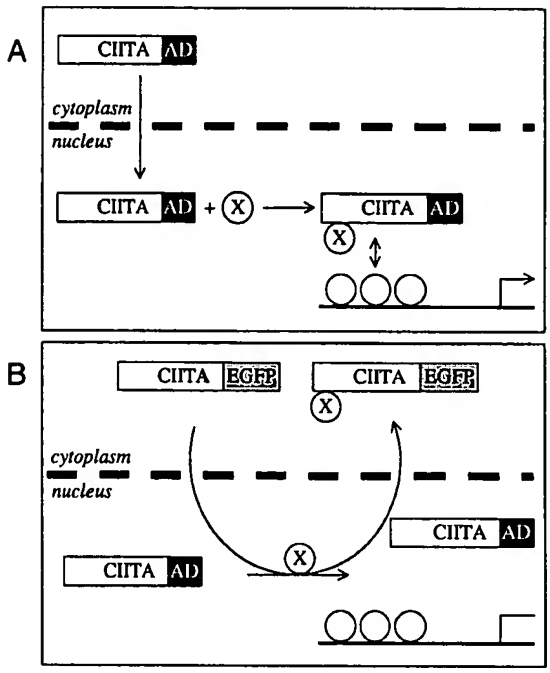

Fig. 8. Model for the blocking of CIITA function by EdCIITA. CIITA orchestrates the transcription of MHC II, Ii and DM genes. It recruits general transcriptional factors, chromatin remodeling machinery and P-TEFb to these promoters. EdCIITA lacks the activation domain of CIITA (positions 1–301) and is found in cytoplasm. Its localization is altered when ΔCAN inhibits the export of EdCIITA from the nucleus, so that although EdCIITA transits the nucleus, it is exported efficiently into the cytoplasm. At steady state, most of EdCIITA is in the cytoplasm. Therefore, EdCIITA most likely sequesters a co-factor of CIITA (X) in the cytoplasm. Other dominant-negative proteins (DNp300 and DNCDK9) then block the activity of the residual endogenous CIITA in the nucleus. Different protein targets and this sequestration of EdCIIITA would explain synergistic effects of these dominant-negative proteins.

facilitated expression and localization studies of this chimera. Surprisingly, EdCIITA worked better than dCIITA and was found in the cytoplasm. dCIITA is a nuclear protein and is present on MHC II promoters in chromatin immunoprecipitations (10). Since it also transits the nucleus, EdCIITA most likely competes with the wild-type protein for the binding to MHC II promoters as well as sequesters a co-factor of CIITA in the cytoplasm. It is possible that the absence of the N-terminal bipartite NLS leaves an unbalanced NES, thereby favoring export of EdCIITA. Alternatively, EGFP itself could favor the cytoplasmic localization, directly or via a conformational change in the fusion protein. Indeed, several other mutations in CIITA, which preserve the N-terminal and C-terminal NLS, keep the mutant protein in the cytoplasm. Importantly, the N-terminal NLS and none of the putative NES have been examined directly for their function.

Second, despite its competition with EdCIITA, the endogenous CIITA can still exert some effects. To this end, we also blocked the ability of CIITA to attract CBP, which remodels chromatin, and of P-TEFb to phosphorylate the C-terminal domain of RNA polymerase II. Indeed, the addition of DNp300 and DNCDK9 functioned synergistically with EdCIITA to extinguish the expression of MHC II genes. As expected, these proteins had to be added separately. A chimera between EdCIITA and DNp300, DNCDK9 or Tat alone or in combination functioned no better than EdCIITA alone. EdCIITA in the cytoplasm and DNp300 or DNCDK9 in the nucleus would then target most efficiently different cellular partners of CIITA. At these lower concentrations of separate components, we would also expect fewer deleterious effects on the transcription of other cellular genes.

The model that emerges from this study is presented in Fig. 8. Since effects of CBP and P-TEFb in MHC II transcription had already been diagrammed, only the sequestration of a critical co-factor for CIITA is depicted. In this scenario, CIITA is a shuttling protein that resides mostly in the nucleus. What circumstance would require its rapid removal from the nucleus via the NES is speculative, but could include aspects of innate immunity, sepsis or toxic shock. With the removal of the N-terminal activation domain and the addition of EGFP, EdCIITA is now a predominantly cytoplasmic protein. However, it transits the nucleus, where it could also compete for the binding to MHC II promoters and remove a critical co-factor from CIITA (protein X). This event might not only inhibit productive interactions between CIITA and MHC II promoters but also affect the communication between CIITA and the general transcriptional machinery. Further details of this mechanism require the isolation and characterization of this co-factor(s). Additionally, strategies of delivering two to three separate dominant-negative proteins efficiently and reproducibly into cells need to be addressed.

Acknowledgements

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Abbreviations

BLS	bare lymphocyte syndrome
CIITA	class II transactivator
CBP	CREB binding protein
CUS	conserved upstream sequences
CREB	cAMP responsive element binding protein
EMSA	electrophoretic mobility shift assay
EGFP	enhanced green fluorescence protein
NES	nuclear export signal
NF-Y	nuclear factor Y
NLS	nuclear localization signal
P-TEFb	positive transcription elongation factor b
RFX	regulatory factor X
SED	streptococcal enterotoxin D

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Aberrant MHC class II expression in mouse joints leads to arthritis with extraarticular manifestations similar to rheumatoid arthritis

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Genetic susceptibility to rheumatoid arthritis (RA) is associated with certain MHC class II molecules. To clarify the role of these determinants in RA, we generated the D1CC transgenic mouse that expressed genes involved in antigen processing and presentation by the MHC class II pathway in joints. The class II transactivator, which was transcribed from the rat collagen type II promoter and enhancer, directed the expression of these genes. In D1CC mice congenic for the H-2^q (DBA/1) background, small amounts of bovine collagen type II in adjuvant induced reproducibly an inflammatory arthritis resembling RA. Importantly, these stimuli had no effect in DBA/1 mice. Eighty-nine percent of D1CC mice developed chronic disease with joint swelling, redness, and heat in association with synovial proliferation as well as pannus formation and mononuclear infiltration of synovial membranes. Granulomatous lesions resembling rheumatoid nodules and interstitial pneumonitis also were observed. As in patients with RA, anticyclic citrullinated peptide antibodies were detected during the inflammatory stage. Finally, joints in D1CC mice displayed juxtaarticular demineralization, severe joint space narrowing, and erosions, which led to ankylosis, but without the appearance of osteophytes. Thus, aberrant expression of MHC class II in joints facilitates the development of severe erosive inflammatory polyarthritis, which is very similar to RA.

autoimmunity | class II transactivator | transgenic mouse | nodules | pneumonitis

Rheumatoid arthritis (RA) is a chronic inflammatory disease with symmetrical inflammatory and erosive polyarthritis of synovial joints and a variety of extraarticular manifestations. Particular MHC class II alleles such as DRB0401, DRB0404, and DQ8 are linked to RA in 30–50% of cases (1–3). To examine possible mechanisms of RA, many models of inflammatory arthritis have been developed in the mouse and rat (4, 5). Among these models, collagen-induced arthritis (CIA) leads to acute inflammation, osteophytosis, and destruction of bone in DBA/1 and B10.Q (H2^q) strains of mice (6, 7). In these mice, passively transferred autoantibodies against CII, or the injection of a combination of certain monoclonal anti-CII antibodies, also induce rapid inflammation of joints (8, 9). A recently discovered model, the SKG mouse, contains mutations in the ζ chain-associated protein of 70 kDa (ZAP-70) that is involved in the signaling from the T cell antigen receptor (10). In these mice, infection with fungi or immunization with zymosan can induce a chronic inflammatory arthritis (11). In K/BxN mice, chronic arthritis develops spontaneously because anti-G6PI antibodies accumulate in serum and joints, leading to inflammatory arthritis and bone destruction (12, 13). In all these mice and several other models, the onset and progression of inflammatory arthritis have been well characterized and analyzed. However, how faithfully these small animal models resemble RA and what roles MHC class II play in their disease have remained elusive.

MHC class II, DM, and invariant chain (Ii) genes are all regulated at the transcriptional level by the class II transactivator (CIITA) (14). These genes share cis-acting sequences (S, X, and Y boxes) in their promoters and enhancers that bind regulatory factor X (RFX) and nuclear factor (NF-Y) that, in turn recruit CIITA (15, 16). CIITA then binds many coactivators that increase rates of initiation and elongation of MHC class II, DM, and Ii genes. RFX and NF-Y are expressed ubiquitously. In sharp contrast, the expression of CIITA is restricted to antigen-presenting cells and mature B cells (17). Thus, CIITA is the master transcriptional integrator that leads to the expression of genes required for antigen processing and presentation by the MHC class II pathway. Interestingly, the cytokine IFN-γ induces the synthesis of CIITA and, thereby, of MHC class II in many somatic cells, which transforms them to "professional" antigen-presenting cells at sites of inflammation (18).

To determine whether the aberrant expression of MHC class II in joints can lead to or potentiate inflammatory arthritis in mice, we linked the human CIITA gene to the rat CII promoter and enhancer (19). This plasmid construction then was used to create transgenic DBA/1, CII promoter/enhancer-driven CIITA (D1CC) mice, which were analyzed further.

Results

D1CC Mice Express MHC Class II in Joints and Are Highly Susceptible to Immunization with Low Doses of Bovine CII (bCII). D1CC mice express CIITA and MHC class II in joints (Fig. 6, which is published as supporting information on the PNAS web site). Although spontaneous swelling and redness would occur in isolated joints of most mice, D1CC mice did not develop chronic symmetrical polyarthritis. Similar to SKG and DBA/1 mice (CIA model), the D1CC mice required a trigger to develop inflammatory arthritis. However, because of the aberrant expression of MHC class II in joints, we expected the D1CC mice to be more responsive to the immunization with bCII than parental DBA/1 mice. Thus, we tested progressively lower doses

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The authors declare no conflict of interest.

Abbreviations: CII, collagen type II; bCII, bovine CII; hiCII, high-dose bCII; loCII, lower doses of bCII; CIITA, class II transactivator; CCP, cyclic citrullinated peptide; CIA, collagen-induced arthritis; CT, computed tomographic; D1CC, DBA/1, CII promoter/enhancer-driven CIITA; RA, rheumatoid arthritis.

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of bovine CII (loCII) in D1CC mice. We used the clinical score in Table 1, which is published as supporting information on the PNAS web site, to monitor the development of inflammatory arthritis (6). Indeed, the D1CC mice developed severe CIA after immunization with 5–10 μg of bCII (loCII-D1CC), a dose that never elicited detectable disease in DBA/1 mice (loCII-DBA/1; Fig. 1*A*). Thus, the conventional DBA/1 control mice required 20- to 40-fold higher amounts of bCII [200 μg of bCII, high-dose bCII (hiCII)] for the induction of CIA (hiCII-DBA/1, Fig. 1*A*).

The incidence of disease between sexes was not statistically different (data not presented). Moreover, in loCII-D1CC mice, the peak clinical score of inflammatory arthritis was the same as in hiCII-DBA/1 mice. However, the inflammatory period lasted 34.3 ± 3.7 days in loCII-D1CC mice versus 15.9 ± 2.7 days in hiCII-DBA/1 mice (Fig. 1*A*). Thus, the duration of inflammation was twice as long in loCII-D1CC mice. The Mann–Whitney *U* test was used to confirm the significance of this difference ($P < 0.01$). In addition, the disease incidence was increased from 57.1% in hiCII-DBA/1 mice to 89% in loCII-D1CC or hiCII-D1CC mice (Table 2, which is published as supporting information on the PNAS web site). Whereas redness and swelling were severe in loCII-D1CC mice, no obvious clinical symptoms were observed in loCII-DBA/1 mice (Fig. 1 *B–E*). We also examined temperatures at various limbs by thermography. Temperatures averaged ≈25°C in loCII-DBA/1 mice and increased up to 28°C in loCII-D1CC mice (Fig. 1 *F* and *G* and thermographs below). Taken together, D1CC mice developed chronic inflammatory arthritis with redness, swelling, and fever at the joint after immunization with minimal amounts of bCII. This finding indicates that the aberrant expression of MHC class II in synovial joints facilitates the development of chronic inflammatory arthritis in the D1CC mouse.

Histological Features of Articular Inflammation in loCII-D1CC Mice. We also examined the progression of disease by histology. At 1 week after the second immunization, there was no inflammation in loCII-DBA/1 mice (Fig. 2 *A* and *D* and data not shown). At 8 weeks, infiltration of inflammatory cells, proliferation of synoviocytes with pannus formation, and erosion of bone were observed exclusively in loCII-D1CC, but not loCII-DBA/1 mice (Fig. 2 *B* and *E*). In the loCII-D1CC mice, the number of infiltrating cells declined dramatically at 15 weeks (Fig. 2*C*). However, an abundance of granulocytes (Fig. 2*C*) and mast cells (Fig. 7, which is published as supporting information on the PNAS web site) had migrated into the pannus at that point, and although the subchondral articular cartilage was severely disrupted at this stage, no subluxation and/or ankylosis was detected until much later. We also found granulomatous lesions resembling rheumatoid nodules in loCII-D1CC but not in loCII-DBA/1 mice (Fig. 2 *G* and *H*).

Interstitial Pneumonitis in loCII-D1CC Mice. We found interstitial pneumonitis with fibrosis as an extraarticular manifestation of disease only in loCII-D1CC but not in hiCII-DBA/1 mice >6 months after the first immunization (Fig. 3 *B* and *D*). There was also a sizeable infiltration of mononuclear cells such as neutrophils and macrophages in these pulmonary lesions. However, because the mice were kept under specific pathogen-free (SPF) conditions and we did not find the deposition of fibrin in D1CC or DBA/1 mice, there was no indication that this inflammation could be due to infection (Fig. 3 *A* and *C*). However, newly synthesized elastic fibers were identified in loCII-D1CC mice by elastica and Kernechtrot staining (Fig. 3*B*). Because interstitial pneumonitis has not been described with CIA but can be found in severe RA, this feature again documents the similarities in inflammatory lesions between loCII-D1CC mice and RA in humans.

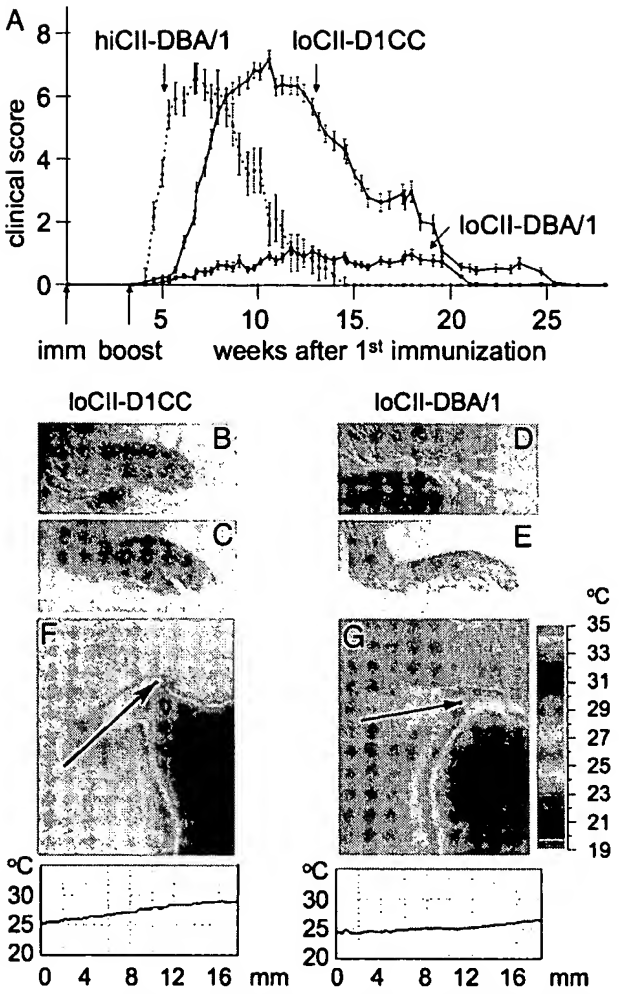

Fig. 1. Chronic arthritis in the loCII-D1CC mouse. To compare D1CC and DBA/1 mice receiving loCII or hiCII, clinical pictures of each mouse were monitored twice weekly and scored (Table 1). Eight-week-old mice were injected with bCII/complete Freund's adjuvant and boosted with bCII/incomplete Freund's adjuvant 3 weeks later (arrows below the graph). (*A*) loCII-D1CC but not loCII-DBA/1 mice develop polyarthritis, which persists for a longer period. Graphs are as follows: Line with filled circles, D1CC mouse injected with loCII (loCII-D1CC); bold line with filled squares, DBA/1 mice injected with loCII (loCII-DBA/1); dashed line with filled triangles: DBA/1 mice injected with hiCII (hiCII-DBA/1). Eighty-nine percent of loCII-D1CC but only 57% of hiCII-DBA.1 mice developed arthritis. Our clinical criteria focused on heat, swelling, redness, and functional impairment. (*B–E*) Joints of loCII-D1CC mouse are swollen. Although parental DBA/1 mice had no phenotype on loCII (*D* and *E*, fore limbs and hind limbs, respectively), loCII-D1CC mice developed swollen and red extremities on this regiment (*B* and *C*, fore limbs and hind limbs, respectively). (*F* and *G*) Joints of loCII-D1CC mouse are hot. Thermographs were performed with joints of loCII-D1CC and loCII-DBA/1 mice. Whereas the maximum temperature at the foot reached 25°C in average in the loCII-DBA/1 mouse (*G*), it reached 28°C on average in the loCII-D1CC mouse at 8 weeks (*F*). Arrows in *F* and *G* point in the direction of thermographs, from arrow point to arrowhead. Numbers below the graph indicate the distance from arrow point in millimeters. The thermal scale with corresponding temperatures (°C) is presented as colors changing from blue to red to the right of *G*.

Bone Destruction and Decreased Mineral Density in loCII-D1CC Mice. Next, we demonstrated the severity of articular pathology by radiography. In loCII-D1CC mice, decreased mineral density was observed at 18 and 39 weeks after the immunization but not at earlier stages of inflammation (Fig. 4 *A–D*). To substantiate

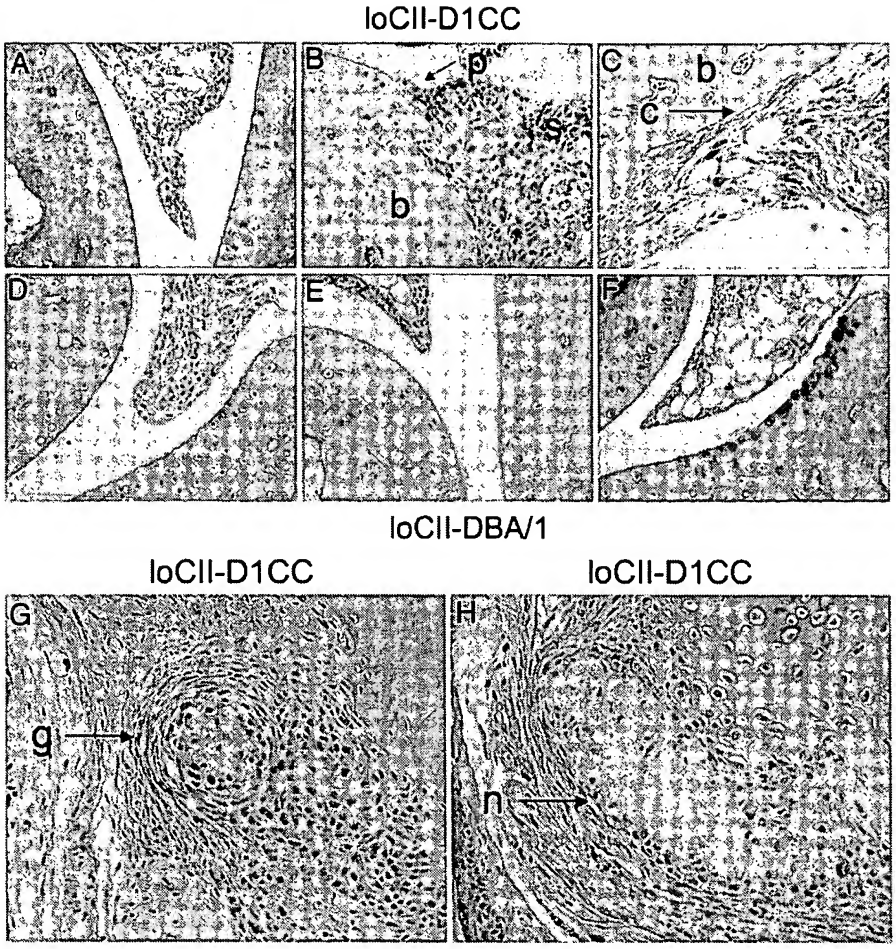

Fig. 2. Histology of the loCII-D1CC mouse. (*A–C*) Histology of joints from the loCII-D1CC mouse. Although no changes are observed 1 week after the boost (*A*), pannus formation over articular cartilage and proliferating synoviocytes (p and s, respectively; *B*), and thinning of articular cartilage (c; *C*) are observed at 8 and 15 weeks after the boost, respectively. Bone (b) also is labeled. (*D–F*) Histology of joints in the loCII-DBA/1 mouse shows no changes at 1 (*D*), 8 (*E*), and 15 (*F*) weeks after the boost. (*G* and *H*) Nodules are observed in the loCII-D1CC mouse. Three weeks after the boost, granulomas (g; *G*) and nodules (h; *H*) are observed only in the loCII-D1CC mouse.

further these radiographic findings, we also performed computed tomographic (CT) scans focused on the large knee joints. Bone destruction, joint space narrowing, and erosions were observed at the patella, distal femur, proximal tibia, and fibula (Fig. 4 *E–J*; see Movies 1–3, which are published as supporting information on the PNAS web site). In addition, relatively dark-gray areas that represent decreased mineral densities paralleled the progression of bone erosions. Osteophytes and "rebound" new bone formation such as arthrodysplasia were found only in hiCII-DBA/1 mice (Fig. 4 *I* and *J* and Movies 1–3) (7). To confirm the osteoporosis at the knee joint, the bone mineral density of cancellous bone in each mouse was calculated from CT scan data. The bone mineral density declined immediately after immunization in hiCII-DBA/1 mice (Fig. 4*K*). It decreased by ≈10% and then recovered at 3 months. In contrast, it decreased by 25% at 3 months and lasted at least 6 months in loCII-D1CC mice. At the terminal stage of chronic inflammatory arthritis, severe ankylosis also was observed at the proximal interphalangeal and metacarpophalangeal joints in loCII-D1CC mice (Fig. 5 *A* and *B*). Even though they were observed for >1 year, we did not detect any osteoporosis or bone erosions in loCII-DBA/1 mice. Thus, the articular deterioration of the loCII-D1CC mouse, in contrast to that of CIA, seems to be chronic and progressive, similar to the joint destruction seen in severe active RA.

Discussion

In this report, we present the D1CC small animal model of inflammatory arthritis. In these mice, the entire machinery for antigen processing and presentation by the MHC class II pathway was activated aberrantly via transgenic expression of CIITA. First, the D1CC mice containing ≈10 copies of the CIITA transgene expressed easily detectable CIITA transcripts and MHC class II in joints (Fig. 6). Second, chronic inflammatory arthritis developed in 89% of male and female loCII-D1CC mice (as contrasted to 57% of CIA in hiCII-DBA/1 mice) after immunization with small doses of bCII that had no effect on parental DBA/1 mice. Third, not only did this arthritis start slower and last longer than CIA in hiCII-DBA/1 mice, but it resulted in characteristic articular and extraarticular manifestations very closely resembling those of severe RA in humans. In loCII-D1CC mice, chronic symmetrical polyarthritis eventually led to ankylosis and destruction of affected joints. Finally, as in RA, D1CC mice developed antibodies to CCP (Table 3, which

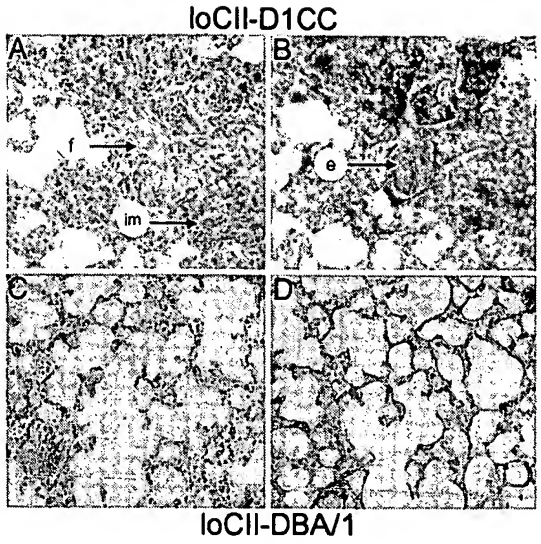

Fig. 3. Interstitial pneumonitis is observed only in loCII-D1CC mice. Histology of lungs 6 months after the boost in loCII-D1CC and loCII-DBA/1 mice. Fibrosis, infiltrating mononuclear cells (f and im, arrow, respectively; *A*), and newly produced elastic fiber (e, arrow; *B*) are observed in lungs of the loCII-D1CC mouse but not in those of the loCII-DBA/1 mouse (*C* and *D*). *A* and *C* were stained with HE. *B* and *D* were stained with elastica and kernechtrot stains to visualize elastic fibers.

is published as supporting information on the PNAS web site). Importantly, unlike other models of inflammatory arthritis (5), we did not introduce any mutations into the mouse. Thus, our work returns the emphasis of RA back to the normal and aberrant immune responses in the host. We propose that D1CC mice may represent an attractive additional animal model for the study of RA.

Histologically, inflammatory infiltrates and proliferating synoviocytes leading to granulomatous lesions resembling rheumatoid nodules were observed only in loCII-D1CC mice. Even though the presence of palisading histiocytes surrounding necrotic nodules could not be ascertained, similar pathology has been reported with pristane-induced arthritis, MRL-lpr/lpr, and SKG mice but not with CIA in DBA/1 mice (11, 20, 21). In contrast to the SKG mice, the D1CC mice had no skin inflammation. Slowly progressive inflammatory arthritis also led to easily identifiable migration of mast cells into the pannus. Because these mast cells expressed MHC class II and B7.1, they might potentially function as antigen-presenting cells (Fig. 7; refs. 22 and 23).

We also observed interstitial pneumonitis with fibrosis and deposition of newly synthesized elastic fibers in the lungs of loCII-D1CC mice. These lesions lacked fibrin, which can be related to infection. Serological analyses revealed the presence of anti-CII and CCP antibodies in sera of both loCII-D1CC and hiCII-DBA/1 mice (Table 3). Notably, the concentration of anti-CCP antibodies was significantly higher in loCII-D1CC than in hiCII-DBA/1 mice, suggesting a more sustained inflammatory process in joints of loCII-D1CC mice. However, attempts to measure the concentration of cytokines and anti-CII antibodies in the synovial fluid were not successful. Radiographic examinations and CT scans revealed further similarities between the arthritis in the loCII-D1CC mouse and RA. These data included periarticular osteoporosis, joint space narrowing, and severe destruction of bone without osteophytosis, all of which led eventually to ankylosis. Although the erosive destruction of bone started in the earlier stages of inflammation at 8 weeks, this bone erosion could only be detected by histology and not yet radiographically (Figs. 2*B* and 4 *A–D* and *K*). Recently, similar microscopic joint damage was detected by MRI in RA patients (24–26).

In a subgroup of RA patients, MHC class II represent a critical genetic risk factor for the susceptibility to the disease (27). Moreover, the expression of these HLAs (HLA class II) have been detected in chondrocytes of RA patients (28, 29). From our study, we hypothesize that the expression of MHC class II on target organs can increase their sensitivity to arthritogenic stimuli in general. This observation might explain the spontaneous inflammation of individual joints without immunization in D1CC mice, some of which persisted for prolonged periods of time and could have resulted from local trauma (data not presented). Moreover, increased sensitivity also might explain the rather severe bone destruction we found in loCII-D1CC mice. In contrast, acute inflammation induces osteophytosis in CIA in DBA/1 mice, where bone resorption is more transient and new bone formation is enhanced soon thereafter (7, 30). Finally, joints in loCII-D1CC but not loCII-DBA/1 progressed to ankylosis and severe functional impairment. We found severe bone destruction in almost all metacarpophalangeal joint and in >60–80% of the proximal interphalangeal joints. These data suggest that lower doses of bCII lead to a more chronic arthritis in the more susceptible D1CC than DBA/1 mice, which is reminiscent of a slow smoldering course of progressive joint destruction seen in severe RA (4, 31).

D1CC mice also provide an excellent breeding partner for other models of inflammatory arthritis in the mouse. Because the balance between Th1 and Th2 was not significantly different in D1CC mice, aberrant expression of MHC class II together with abnormal selection of T cells such as survival of autoreactive clones will be investigated further. For example, the hCIITA transgene also might influence other types of arthritis in mice and possibly render the arthritis in other animal models, such as SKG mouse, spontaneous (20, 32, 33). Moreover to create even better and more mouse models of RA, D1CC mice may be backcrossed with the DRB1*0401 or DRB1*0402, and human CD4 double-transgenic mice, which lack I-A$\beta^{-/-}$ on the DBA/1 background (34, 35). Both DR genes are expressed from the mouse I-Eα promoter and enhancer, thus aberrant CIITA expression will induce the expression of DR*0401 or DRB1*0402 in their chondrocytes (36). DRB1*0401 is not only one of the susceptible genes for RA but is also the molecule that presents citrullinated arthritogenic antigens. However, DRB1*0402 does not present the same peptides. Thus, these DR alleles will help us to dissect the contributions between specific arthritogenic stimuli and MHC class II from RA patients. Also, specific peptides that are presented by aberrantly expressed MHC class II in humanized D1CC mice will be used to tolerize these mice to these antigens, which then can be evaluated for effects of such therapeutic vaccination on their disease.

Materials and Methods

CIA. For the induction of CIA, D1CC and DBA/1 mice at 7 to 9 weeks of age were housed in a pathogen-free animal care facility of Nagoya City University Graduate School of Medical Sciences in accordance with institutional guidelines. These mice were anesthetized with diethyl ether before induction of CIA. At that point, they were immunized with bCII (5–10 μg, called loCII for D1CC and DBA/1 mice and 200 μg, called hiCII for DBA/1 mice) (Collagen Research Center, Tokyo, Japan), which was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Mice were injected intradermally at the base of the tail, near inguinal and axillary lymph nodes on day 0 as the first injection. On day 21, the mice were boosted with the bCII in the same manner except that incom-

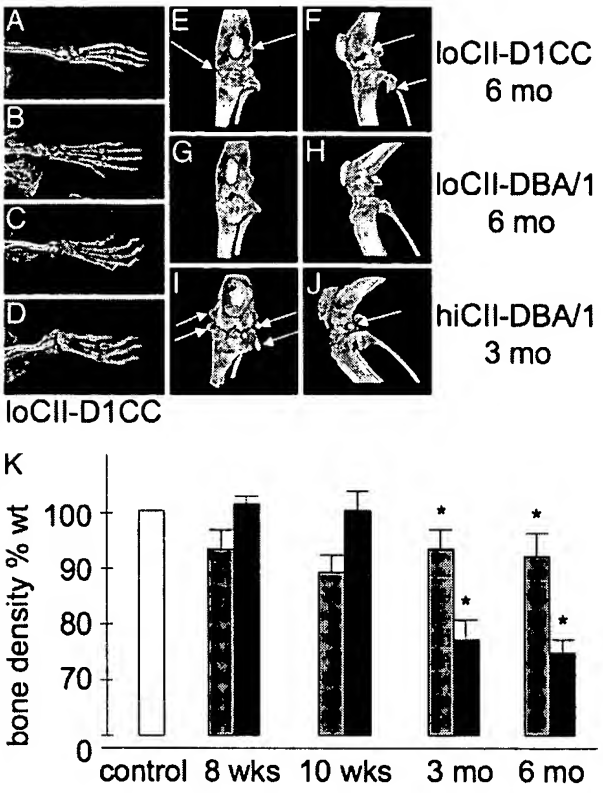

Fig. 4. Joint space narrowing, erosions, and osteoporosis in the loCII-D1CC mouse. (*A–D*) Progressive joint damage in the loCII-D1CC mouse. Radiographic examination reveals the decline of bone mineral density and joint space narrowing and erosions in the fore limbs at 0 (*A*), 9 (*B*), 18 (*C*), and 39 (*D*) weeks after the first injection. (*E–J*) Joint space narrowing, erosions, and osteoporosis in the knee of the loCII-D1CC mouse 24 weeks after the boost. (*E* and *F*) CT scans are presented for the distal femur, the knee joint, the proximal tibia, and the fibula. Note the narrowing of the joint space (*E*, left arrow), erosions, and osteoporosis (*E* and *F*, right arrows). None of these changes are observed in the parental loCII-DBA/1 mouse treated likewise (*G* and *H*). In sharp contrast, hiCII-DBA/1 mice developed erosions and formed new bone resembling osteophytes 12 weeks after the boost (*I* and *J*, arrows). (*K*) Progressive osteoporosis in the loCII-D1CC mouse. Presented is the comparison of decreased periarticular bone mineral density over time between ldCII-D1CC and hiCII-DBA/1 mice. Ten sections of cancellous bone measuring 0.1 mm each were analyzed by densitometry of CT scans from both knees in each mouse. Times after the boost are presented below the bar graphs. Data are presented as percentage of bone mineral density of loCII-D1CC (black bars) and hiCII-DBA/1 (gray bars) compared with the untreated parental DBA/1 mouse (WT was given the value of 100%; open bar) over time. Six mice were studied in each group. Error bars reflect SEM. All values were calculated by using data from three or four mice. Student's *t* test was performed for each value between loCII-D1CC and hiCII-DBA/1 mice (*, $P < 0.05$).

plete Freund's adjuvant (Difco Laboratories) was used as the solvent.

Evaluation of Joint Arthritis. DBA/1 and D1CC mice were monitored from the onset of disease to the end of active inflammation twice weekly. The clinical severity of arthritis was quantified according to this simple scoring system: 0, no clinical symptom; 1, swelling and redness of one or two joints; 2, moderate swelling and redness of more than three joints; 3, severe swelling and redness of entire paw (6). All scores were added to the total clinical score, which reached the maximum at 12 (four severely affected joints, 4 × 3). The onset of arthritis was defined as the

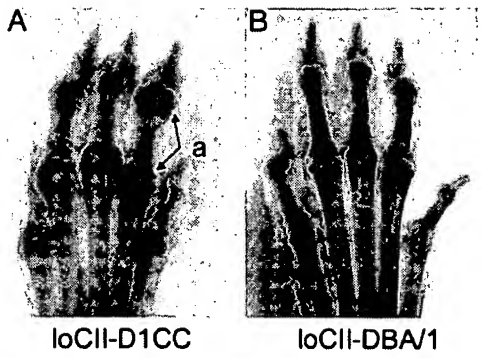

Fig. 5. Fore limbs of the loCII-D1CC mouse reveal extensive joint destruction. Fifteen months after the boost, fore limbs of loCII-D1CC (*A*) and loCII-DBA/1 (*B*) mice were stained with Alizarin and Alcian blue, which stain bone red and cartilage blue. Note the complete destruction of all joints (ankylosis, a, arrows) and shortening of phalanges in the loCII-D1CC mouse and normal joints in loCII-DBA/1 mice.

number of days between the first immunization with bCII and the occurrence of arthritis with a clinical score of >3. The end of acute arthritis was noted when the clinical score was reduced to 3. Body temperatures were measured at all extremities by using the thermograph (Neo Thermo; Nippon Anionics, Tokyo, Japan).

Immunohistochemistry. For immunohistochemical staining, the avidin–biotin–peroxidase complex (ABC) method was used according to protocols from the manufacturer (Vector Laboratories, Burlingame, CA) (37). Paraffin sections were rehydrated and washed with tap water for 5 min. The endogenous peroxidase activity was inhibited by first incubating samples in 0.05% of H_2O_2 solution for 30 min and then washing with PBS for 10 min at room temperature. Thin sections were incubated with buffer C (1.5% normal serum in PBS) for 60 min, and primary antibodies (biotin-conjugated mouse anti-mouse I-A^q monoclonal antibody, BD Biosciences, San Jose, CA) were diluted to 1:6 with the blocking buffer (supplied by Vector Laboratories) for 30 min and washed with PBS for 10 min. All incubation steps with antibodies were performed in a humid chamber. Signals were detected by using a commercial ABC kit and DAB solution (Mouse on Mouse Immunodetection kits PK-2200 and SK-4100, respectively; Vector Laboratories). All specimens were counterstained with hematoxylin and mounted in VectaMount (H-5000; Vector Laboratories). As the negative control, isotype-matched antibodies (biotin-conjugated mouse IgG_{2b}, monoclonal Ig isotype control; BD Biosciences Pharmingen) were used.

Radiographic Technique. The computed radiographs (CR) were obtained by using the CR console (FCR5000plus; Fujifilm, Tokyo, Japan) and the high-resolution CR cassette (Fujifilm). The source of x-rays was a conventional radiographic unit and exposure factors were 50 kV, 50 mA, and 40 ms (Shimadzu, Kyoto, Japan).

Bone Mineral Density and CT. We used peripheral quantitative computed tomography with a fixed x-ray fan beam of 10-mm spot size, at 1 mA and 50kVp (LaTheta LCT-100S; Aloka, Tokyo, Japan). Eighty slices (480 × 480-pixel matrix per slice, 0.1 mm thickness, a voxel size of 65 × 65 × 65 μm^3) covered the entire knee joint and recreated the 3D CT picture of the joint by using the VGStudio MAX1.2 software (Nihon Visual Science, Tokyo, Japan). To measure the mineral density of bones at the joint

from each mouse, data were recalculated from the distal femur and the proximal tibia, both adjoining the articular cartilage.

Histochemical Staining. For H&E staining, paraffin sections were rehydrated and immersed first into the Mayer's hematoxylin solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 5 min and then into the eosin solution (Muto Pure Chemicals Co., Ltd.) for 2 min. For elastical and kernechtrot staining, paraffin sections also were rehydrated. After washing with 1% of hydrochloric acid and 70% ethanol for 5 min, sections were incubated with resorcin fuchsin solution (Muto Pure Chemicals Co., Ltd.) for 60 min, washed with ethanol, and counterstained with Kernechtrot stain solution (Muto Pure Chemicals Co., Ltd.) for 2 min. For toluidine blue staining, sections were rehydrated and washed with tap water for 5 min and incubated with 0.05% of toluidine blue O solution (Waldeck, Muenster, Germany) for 10 min. All sections were dehydrated with ethanol and mounted in VectaMount. To count the number of infiltrated mast cells in the joint, the five most-visible areas of mast cells were selected in toluidine blue stained histological slides. The number of mast cells then were counted in all extremities in D1CC and DBA/1 mice and visualized in a bar graph. For cartilage and bone staining, specimens were immersed in 95% of ethanol for >5 days and in acetone for another 2 days. Skin, muscle, and fat were removed carefully and stained with Alcian blue (0.3% of Alcian blue 8GX; Sigma–Aldrich, St. Louis, MO) and Alizarin red (0.1% Alizarin sodium monosulfonate; Sigma–Aldrich) for 3 days. Finally, specimens were immersed in the 1% KOH solution and glycerol for 2 days.

Statistical Analyses. All measurements were performed in duplicate, and all experiments were repeated at least three times. All error bars give SEM. Mann–Whitney U test was used for the statistical analysis of disease-related parameters between control and arthritic mice. The histomorphometric data and the serum titers of anti-CII antibodies between the control and the arthritic mice were compared by Student's t test. Values of $P < 0.05$ were considered to be statistically significant.

Supporting Information. Additional data can be found in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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